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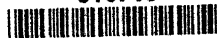
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## ERRATA AND AUTHOR'S EMENDATIONS FOR VOLUME 35, JOURNAL OF AGRICULTURAL RESEARCH

Page 10, fourth paragraph in summary should be deleted.

Page 32, line 7, "8°" should be "82°."

Page 55, line 2, "heterozygous" should be "homozygous."

Page 55, line 3, "contrary to" should be "in accordance with"

Page 203, line 12, "(12)" should not be italicized

Page 319, the box heads of the last two columns at bottom of page should be interchanged

Page 343, Table 10, total dry weight, bottom of column 5, should be ".78" instead of "1.78."

Page 343, Table 10, total dry weight, bottom of column 8, should be ".71" instead of "1.73 "

Page 354, Figure 2, add to legend "is a composite diagram from orchard and laboratory observations."

Page 429, footnote 1, line 3, insert "the Department of Zoology" before "Johns Hopkins University."

Page 448, Table 22, footnote symbol "a" should follow "and water" on second line of table heading.

Page 473, legend of Figure 3, "evaginated" should be "invaginated."

Page 480, last line, for "Hall" read "Liberty Hall."

Page 559, Table 2, column 8, line 19, "1.052" should be "0.052"

Page 566, lines 24 and 25, "sheep No. 202" and "sheep No. 201" should read "guinea pig 202" and "guinea pig 201."

Page 632, footnote at bottom of table refers to previous column in each instance—i. e., to the columns for "Ca in urine and feces," "P in urine and feces," and "N in urine and feces"

Page 638, Table No. 1, last number in column headed "Specific gravity" should be "1.0265" instead of ".0265"

Page 639, Table 2, in footnote d, "2 days" should be "3 days"

Page 639, Table 3, column 1, date should be "February 26" instead of "February 21."

Page 658, legend B for Plate 1 should be "Tobacco mosaic on tobacco. X-body (*b*) and nucleus (*n*) in a hair cell. (Nucleus slightly out of focus.)"

Page 830, Table 2, line 3 in third column from right, "4 89" should be "4 80"

Page 844, paragraph 4, line 4, change "B" to "C."

Page 863, line 9, should be "is of low biological value" instead of "if of low biological value."

Page 869, Table 1, row 131, "142" should be "412"

Page 870, Table 1, rows 599 and 710, place entire C I. Nos. in the column.

Page 914, line 19, "barley in large numbers may not be" should be "barley in large numbers may now be"

Page 1034, Table 8, quality of sauerkraut in vats 3 and 1 should be "fair" instead of "poor"

Page 1034, Table 8, quality of sauerkraut in vats 6 and 4 should be "poor" instead of "fair"

Page 1124, line 27, "suggestions of the" should be "the suggestions of"

Page 1127, lines 4 and 8, change "*batatis*" to "*batatatis*."

Page 1132, Figure 5 is upside down.

Page 1144, fourth line from bottom, delete comma after "considered!" and insert comma after "curvilinear."

Page 1145, line 21, after "protein content" delete "for each 1 per cent increase in dark, hard, and vitreous kernels."



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WASHINGTON, D. C., JULY 1, 1927

No. 1

## FACTORS AFFECTING CERTAIN PROPERTIES OF A MOSAIC VIRUS<sup>1</sup>

By H. H. McKINNEY

*Pathologist in Charge of Cereal Virus Disease Investigations, Office of Cereal Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture*

### INTRODUCTION

Reports on the mosaics of the winter cereal crops (16, 18)<sup>2</sup> indicate that it is difficult to make a systematic study of the virus and of certain important phases of the diseases. That the grass mosaics, under present conditions, are not suitable for use in many of the important fundamental studies of the viruses and of the diseases seems to be very evident. In the case of the winter cereals and corn a large part of this difficulty is accounted for by the fact that these crops do not develop normally under ordinary greenhouse conditions, or in the field, out of their natural growing seasons. Moreover, the virus extracts of all grass mosaics seem to lose their potency rather rapidly after they are expressed by the methods now employed.

A study of the literature on the mosaic diseases of the dicotyledons shows that many of the difficulties which are associated with the grass group are not important and do not constitute serious obstacles in the study of certain mosaics of dicotyledonous plants. As a result, much experimental work has been possible on the properties of the viruses of such mosaics as those of tobacco, cucumber, and tomato.

Practically no work has been done on the properties of the viruses of the grass mosaics. No study has been made of filtration and nothing is known of the tolerance of these viruses to temperature and chemicals, except that inert mineral oil (6) and acetone (9) are claimed to favor the extraction of potent virus from mosaic-affected sugar cane.

In connection with the writer's studies on winter-wheat mosaic, it became necessary to determine whether there was justification for carrying out laborious experiments on the wheat plant and the virus in order to reach the point where the properties of the virus might be studied according to the current methods employed in studying the virus or viruses of the tobacco and cucumber mosaics. There was considerable doubt as to whether the results of similar investigations of the properties of the virus of wheat mosaic would be sufficiently trustworthy or significant to warrant the extensive preliminary studies necessary. Moreover, it seemed evident that the proper understanding of the grass mosaics and their viruses depended

<sup>1</sup> Received for publication Nov. 30, 1926; issued July, 1927. Cooperative investigations between the agricultural experiment station of the University of Wisconsin and the Office of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture.

<sup>2</sup> Reference is made by number (italics) to "Literature cited," p. 11

on the solution of certain basic problems which might never be solved to best advantage on the grasses.

Some time ago it was pointed out (17) that an investigation should be made to determine the influence of plant extracts on the behavior of viruses. At that time it seemed very doubtful if the differences in the properties of virus extracts obtained from different species of plants, as determined by present methods, could be considered specific for the viruses themselves, and it appeared evident that the value of property studies depended to a great extent on the development of methods which would make it possible to obtain a virus in a more nearly pure state.

The literature indicates that the properties of microorganisms and certain biochemicals are greatly affected by the nature of the dispersing medium and by other factors, such as the number of organisms and the concentration of the biochemical. It seemed entirely possible, therefore, that the properties of such viruses as those of cucumber mosaic (7) and tobacco mosaic (1) might be explained wholly or in part on the basis of differences between the extracts of the two species of plants and not necessarily on the basis of any specific differences between the viruses.

The writer (17) pointed out the desirability of testing this theory by studying the properties of a given virus after passage through different species of plants, and, after an absence of several months from the Wisconsin Agricultural Experiment Station, planned to carry out such experiments on his return to that station. However, after this report (17) was submitted it was found that similar studies were just being started at Wisconsin by M. N. Walker. In view of this fact the writer modified his original plans so as not to include the cross-inoculation phases. Accordingly, all of the inoculations reported in this paper were confined to one species of plant, namely, tobacco, and the studies on the effect of plant extracts on the virus were carried out *in vitro*.

#### REVIEW OF LITERATURE

Bigelow and Esty (5), Esty (11), and Esty and Williams (12) have shown that the thermal death points of the spores of many thermophilic bacteria isolated from canned foods are influenced by the number of spores, the hydrogen-ion concentration of the medium, and the nature of the medium other than its hydrogen-ion concentration.

Bigelow and Esty (5) show in their Tables 4, 5, and 6 that the thermal death points of spores of 19 different thermophilic bacteria are affected by the concentration of the spores when all other factors are uniformly alike. One case, typical of all, may be cited. When the spores of organism No. 26 were heated at 120° C. it was found that the several populations per cubic centimeter were killed in the following periods of time: 40,000 in 12 minutes, 3,800 in 10 minutes, 440 in 9 minutes, and 130 in 7 minutes.

Esty and Williams (12) in their Table 1 show that 100,000 spores per cubic centimeter of organism No. 1518, heated at 110° C. in a phosphate mixture of different  $P_H$  concentrations, were affected in the following manner: All spores in from 60 to 63 per cent of the test tubes were killed in 20, 22, 40, and 60 minutes when the  $P_H$  concentrations were 5.10, 5.55, 6.20, and 7.09, respectively.

Bayliss (3), Euler (13), and others point out that the properties of enzymes also are affected by their concentration. Euler states that the action of such paralyzers as chloroform depends to a great extent on the concentration of the enzyme. It is considered that the injurious effects on the enzyme are greater as its concentration decreases. Bayliss and also Euler point out that the properties of enzymes are influenced by colloidal impurities, salts, and proteins present in the extracts. O'Sullivan and Thompson (21) found that sucrose extract is completely inactivated near 55° C. in the absence of sucrose, but when sucrose is present the enzyme was only partially inactivated near 75°. Bayliss (2) found that the presence of charcoal in an extract of trypsin increased the thermal tolerance of the enzyme.

Wells (23) indicates that the properties of toxins and antitoxins are affected by many substances and that a given property of a toxin or antitoxin is not necessarily fixed under all conditions.

From these citations it is evident that some of the properties of microorganisms and of biochemicals do not remain constant under all conditions. Thus the assumption that the properties of a virus may be affected by its concentration and by constituents in the extract has some support. To test this assumption, the following experiments were conducted with the virus extract from tobacco affected with mosaic: (1) Determination was made of the effect of the virus concentration on (a) the thermal inactivation point and (b) the potency of the virus while stored at room temperature; (2) the effect of such diluting media as water and cucumber and tobacco fluids was determined on thermal inactivation and on potency of the virus while standing at room temperature.

#### METHODS

The virus was obtained originally from a single young mosaic affected tobacco plant which was kindly supplied the writer by James Johnson and M. Mulvania. The mosaic pattern on this plant<sup>3</sup> was comparable to that illustrated by Iwanowski (14) and, although the mottled leaves showed no deformities, many of the plants subsequently inoculated with this virus developed leaf deformities similar to those illustrated by Iwanowski.

This plant was handled with great care to avoid any contamination with other viruses. The hands were washed in 95 per cent alcohol and dried immediately before the plant and the fluid were handled. The plant was ground to a pulp in a sterilized food chopper. The pulp was collected in sterile gauze placed in a sterile dish and the fluid was then pressed out by hand. Part of the fluid was put in a sterile test tube. This was put away and safeguarded against any

<sup>3</sup> It has been pointed out by the writer in a brief article (19) that the virus of this plant regularly causes small, yellow spots on older plants affected by the typical "green mosaic." The virus obtained from these isolated yellow spots produces "yellow mosaic" in young plants, and by successive subtransfers of virus from yellow spots the proportion of yellow mosaic can be increased to a point where it dominates over the green mosaic throughout the complete life cycle of the plant.

Five additional lots of tobacco-mosaic virus were obtained from widely separated regions in the United States, and another lot was obtained through R. J. Eiker from England. When plants were inoculated with these viruses it was found that the same type of yellow spots developed in older plants and that all were like the spots described above. In one case fresh leaves which were especially well preserved while in transit, showed a yellow spot on being received from the sender. This spotting usually develops when the plant has produced from 12 to 15 leaves. All of the evidence now in hand indicates that the association of these viruses may not constitute a contamination in the usual sense. A more complete report will be made of this work later.

possible contamination from another virus. The few plants which were inoculated in the beginning supplied virus for subsequent experiments.

In all experiments the virus and plant extracts were obtained by grinding mosaic-diseased or mosaic-free plants in a mortar with quartz sand. The fluid was obtained by pressing the pulp in a screw press or by hand. Although the fluids were not filtered, all coarse tissue was removed by passing the fluid through several thicknesses of finely woven cotton gauze. Dilutions were made immediately with sterile distilled water or with fresh fluid from mosaic-free tobacco and cucumber plants. Dilutions were not carried beyond a point (one thousand times) which would produce 100 per cent infection with fresh unheated infectious juice. All glassware, grinders, gauze, or other materials which came in contact with the virus were sterilized before being used.

In all of the experiments on thermal inactivation, the extracts were placed in pieces of glass tubing which were 7 mm. in diameter (inside) and 45 mm. long, 0.8 c. c. of extract being placed in each tube. The tubes were closed at both ends by means of small, tightly fitting corks. These were held tight by means of a stout cord. The temperatures were maintained by an insulated water bath 6 inches deep and 8 inches in diameter, and the temperature was determined by means of a standardized thermometer. The temperatures were regulated within less than  $0.2^{\circ}$  C. by means of a hand-regulated gas flame. The tubes were completely immersed and held in the center of the bath on a wire tray, which also served as an agitator. The temperature of the water was taken at the level of the tray. Fifteen seconds were allowed for the extracts to reach the temperature of the water. As soon as the tubes had been subjected to the proper temperature for the prescribed time, they were all removed at once and plunged into cold water immediately.

This method was adopted after a study had been made of the methods commonly employed. It is natural to expect greater regularity in heat penetration of substances in small containers which are completely immersed than in test tubes which are not completely immersed. This point seems to be borne out by Bigelow's (4) studies on heat in the canning industry. Most bacteriologists use ampules of small diameter, drawn and sealed at the ends in a gas flame. The writer employed this method, but it was discarded and the corked-tube method was substituted. The continued use of the method has proved it to be very convenient and simple for studies of viruses, as temperatures do not go above  $100^{\circ}$  C.

In the studies on the reduction of the potency of the virus while standing at room temperature, each lot of virus extract was diluted with the prescribed quantity of sterile, distilled water, or tobacco or cucumber extracts. The resulting fluids were put in sterile test tubes, which were then corked and stored in the laboratory. This method might have been refined somewhat by using filtered fluids. However, as unfiltered juices have commonly been employed by those studying the properties of viruses, and as quantitative studies (20) show that filtered juice usually has a very low virus concentration, it was decided to use unfiltered fluids in these experiments.

In all of the experiments the plants were cultured and inoculated in accordance with the methods described in the accompanying

paper (20). The writer kept close watch for any variation in symptom manifestations which might be caused by heating or diluting the virus. The mosaic symptoms of different plants which have been subjected as nearly as possible to the same conditions do vary somewhat, and there is also some variation due to different environmental conditions, but, after taking these variations into account, the writer has not yet seen additional symptom variations which seemed to result from the treatments of the virus. In some cases mild forms of mosaic appear in the early stages of the disease. However, in all such cases resulting from heated virus, the writer has held the plants for a longer period, and in every instance such plants have soon developed typical mottling of a rather severe type. All of the evidence obtained thus far clearly indicates that critical studies can not be made in mosaic symptomatology entirely on young stock. Mosaic-affected plants must be held for relatively long periods, sometimes until flower-bud development, if a complete story of symptom types is to be obtained.

Some investigators object to holding plants for long periods because of the possibility of their becoming accidentally infected with other viruses. This objection is begging the question. Precautions must be employed which make it safe to follow this method.

## RESULTS

The data obtained in the studies on thermal inactivation are presented in Tables 1 to 4. From these data it will be seen that at the higher temperatures the potency of the virus becomes less as the concentration of the virus is reduced. It will be seen also that this general relationship holds consistently for all of the diluting media used. However, it is evident that the degree of reduction in the infectious activity was influenced by the diluting medium. In all experiments the uninoculated control plants remained free from mosaic symptoms.

TABLE 1.—Percentage of tobacco plants that developed mosaic in three experiments in each of which five plants were inoculated with virus extract undiluted or diluted 10, 100, or 1,000 times with sterile distilled water, and held at 80° or 85° C. for different periods

Time virus was exposed to 80° C.	Percentage of mosaic at dilutions of—			Time virus was exposed to 85° C.	Percentage of mosaic at dilutions of—		
	0	100	1,000		10	100	1,000
5 minutes.....	100	100	60	5 minutes.....	100	80	60
20 minutes.....	100	100	0	10 minutes.....	100	60	20
Unheated control.....	100	100	100	15 minutes.....	100	20	0
5 minutes.....	100	100	100	20 minutes.....	100	0	0
20 minutes.....	100	80	40	Unheated control.....	100	100	100
Unheated control.....	100	100	100				

In some cases, heated virus extract diluted one hundred times caused 100 per cent infection. However, except in one case, infection was reduced by heating virus diluted one thousand times. The one exception is shown in Table 1, where the virus was heated for but five minutes at 80° C. It is not surprising that 100 per cent infection

was produced by some of the heated extracts diluted one hundred times, as the unheated virus diluted one thousand times always produced 100 per cent infection, except when cucumber fluid was used. Furthermore, heat treatments at 80° do not appear to be sufficiently severe to produce marked injury with regularity. Doubtless quantitative tests would have shown that the potencies of the viruses in the dilutions of one hundred times and in the exception cited above were actually reduced by the heat treatments, even though this reduction was not sufficient to show in these tests.

TABLE 2.—Percentage of tobacco plants that developed mosaic when five plants were inoculated with virus extract undiluted or diluted 100 times with sterile distilled water, and held for 10 minutes at different temperatures

Temperature for 10 minutes (°C.)	Percentage of mosaic at dilutions of—		Temperature for 10 minutes (°C.)	Percentage of mosaic at dilutions of—	
	0	100		0	100
82.....	100	40	90.....	0	0
84.....	100	0	92.....	0	0
86.....	100	0	Unheated controls.....	100	100
88.....	80	0			

TABLE 3.—Percentage of tobacco plants that developed mosaic when five plants were inoculated with virus extract undiluted or diluted 10 or 100 times with sterile distilled water or with tobacco extract <sup>a</sup>

Temperature for 10 minutes (°C.)	Dilution magnitudes and percentages of mosaic					
	Water dilutions			Tobacco-fluid dilutions		
	0	10	100	0	10	100
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
80.....	100	100	40	100	100	100
84.....	100	100	20	100	80	80
88.....	100	0	0	100	40	0
Unheated controls.....	100	100	100	100	100	100

<sup>a</sup> Although the plant from which this fluid was obtained showed no signs of mosaic, the fluid contained a very small trace of virus, as one out of five control plants inoculated with this undiluted fluid developed mosaic.

TABLE 4.—Percentage of tobacco plants that developed mosaic when five plants were inoculated with virus extract undiluted or diluted 10, 100, or 1,000 times with tobacco or cucumber extract

Temperature (°C.) for 10 minutes	Dilution magnitudes and percentages of mosaic						
	Tobacco-fluid dilutions				Cucumber-fluid dilutions		
	0	10	100	1,000	10	100	1,000
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
85.....	100	100	20	0	0	0	0
Unheated controls.....	100	100	100	100	60	40	0

In order to obtain some idea of the influence of the virus concentrations on the temperature at which inactivation occurs, an experiment was carried out with fresh, undiluted extract and with the same extract diluted one hundred times in water. This dilution is very low and has never failed to produce 100 per cent infection. These extracts were then subjected to a series of 10-minute treatments at temperatures ranging from 82° to 92° C. The results shown in Table 2 indicate that the thermal inactivation point dropped at least 5° when the virus was diluted one hundred times. It is fully recognized that the extract in each dilution varied with the concentration of the virus, and this doubtless had some influence on the results. However, this point can not be accurately determined until it is possible to study a purified virus in a uniform extract.

A comparison of the data in Tables 1 and 2 shows that the virus extract used in the 85° C. heat series, which was diluted one hundred times, caused 60 per cent of mosaic when heated 10 minutes (Table 1), whereas the virus of the same dilution when heated at 84° and 86° for 10 minutes caused none (Table 2). This difference in the behavior of the virus can not be explained at the present time. It is possible that the two viruses differed considerably in their initial concentrations. It is also likely that the fluids in the different plants varied. Observations show that the physical and chemical nature of the fluids vary greatly in plants grown under different conditions. The coagulation temperature of the proteins in the fluid seems to vary considerably, and the precipitation of coagulated materials also varies greatly. Any or all of these variables doubtless affect the thermal inactivation and other properties of the virus. It therefore becomes apparent that attention must be directed toward the more accurate control of growth conditions in order that reasonably uniform plant fluid may be obtained.

From the data presented in Table 4 it is noted that dilution with fluid from healthy cucumber plants had a very depressing effect on the thermal inactivation of the virus of tobacco mosaic. Cucumber fluid also reduced the infectiousness of the unheated virus. From these results it seems entirely possible that the relatively low thermal inactivation point of the virus of cucumber mosaic may be explained in part on the basis of the nature of the cucumber-plant fluid.

The experiments dealing with the effect of time and of different diluting media on the potency of the virus were not repeated as many times as were the experiments relating to temperature and dilution. However, in general, the results seem to be sufficiently consistent to warrant presentation in Table 5.

From these data it is apparent that after standing, the virus became less infectious as its concentration was reduced. In general, it appears that the healthy tobacco and cucumber fluids, especially the latter, had a little more depressing effect on the potency of the virus than did sterile distilled water. These results and those in Table 4 make it seem that the short period over which the expressed virus of cucumber mosaic retains its infectious property also may be due in part to the nature of the cucumber-plant fluids.

TABLE 5.—Percentage of tobacco plants that developed mosaic in four experiments in each of which five plants were inoculated with unheated virus extract undiluted or diluted 10, 100, or 1,000 times, with distilled water, tobacco, or cucumber extracts <sup>a</sup>

Time (days) virus stood in laboratory	Dilution magnitudes and percentages of mosaic									Un- diluted virus
	Water dilutions			Tobacco-fluid dilutions			Cucumber-fluid dilutions			
	10	100	1,000	10	100	1,000	10	100	1,000	
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	
2.....	100	80	60	100	80	20	100	20	0	100
6.....	100	100	80	100	100	80	100	80	0	100
12.....	100	80	80	80	60	40	80	60	0	100
15.....	100	40	40	100	40	20	100	40	20	100
Means.....	100	75	65	95	70	40	95	50	5	100

<sup>a</sup> The dilution samples stood in the laboratory in corked test tubes during the period of the experiments.

It is noted from Table 5 that the virus diluted more than ten times tended to cause a higher percentage of infection after it had stood for six days than it did at the end of the second day. When the virus was diluted one thousand times in cucumber fluid the infection was greatest after 15 days' standing. In general, after the sixth day the infection curves for virus diluted above ten times tended to drop.

#### DISCUSSION

In somewhat similar experiments, Elmer (10) observed that infection from diluted virus of tobacco mosaic was first depressed and later increased. However, he worked only with filtered fluid and with dilutions of ten times. Also, his studies were conducted over a shorter time period than were the writer's. In the present studies, the plant fluids had little effect on the virus when diluted ten times. Elmer's results with virus diluted ten times are probably explained in part on the basis of a very low initial concentration of the virus. It seems likely that the virus used in Elmer's experiments was from fifty to one hundred times less concentrated than virus employed in the present studies.

Indications of an accelerating or reactivating influence on the potency of the virus has been evident in several of the writer's experiments. However, it appears now that much study is necessary before it will be possible to distinguish with certainty between what may be experimental variation and actual reactivation of previously inactivated virus through colloidal and adsorption changes, or through an actual increase in the number of virus particles not previously inactivated.

Granting for the moment that this apparent reactivation of the virus may not be due entirely to experimental variation, it is of interest to consider the behavior of certain biochemical substances. From a review of the literature on enzymes and toxins, it appears that these substances vary greatly in their ability to dissociate or disperse as the adsorption relationships are altered in their extracts. There is considerable evidence which indicates that inactivation

does not necessarily signify destruction. Under certain conditions, these substances may be reactivated after having been inactivated, or partially inactivated. The expressed viruses of plants are associated with many other materials in the fluid, and when the fluid is allowed to stand it undergoes many changes. Under such conditions the reaction and adsorption relationships doubtless are altered many times, and it seems quite likely that these fluctuations may affect the quantities of free virus from time to time. While it may be that we are dealing with an organism which, under certain circumstances, increases *in vitro*, it seems apparent that it is not necessary to consider that the virus is an organism in order to explain the results cited.

Duggar and Armstrong (8) have reported that the fluids from the pokeweed inhibit the infectiousness of the virus of tobacco mosaic *in vitro*. They tested the effect of the juices from many plants on the virus and found that some of them did not seriously reduce its infective power. The writer carried out similar experiments with pokeweed juice and obtained the same results. In addition, several tests were made in which tobacco plants were inoculated with the fresh virus extract of tobacco mosaic, and soon afterward the same plants were reinoculated with the fresh extract from healthy pokeweed plants. In another set of experiments the plants were first inoculated with the pokeweed juice and then inoculated with the virus extract. In all of these cases, when the pokeweed and virus extracts were not mixed *in vitro* before making the inoculations, mosaic occurred in 100 per cent of the inoculated plants.

It is not surprising that certain plant extracts are toxic to a virus *in vitro*. Our most deadly poisons in many cases are of plant origin and it would be indeed surprising if some of these are not found to be very toxic to many substances in other species of plants. Only by further studies can it be determined if the toxic principle is in the pokeweed juice or if there is a new or secondary substance produced by a combination of substances in the pokeweed and tobacco extracts which is toxic to the virus.

Since the preparation of the present paper, the results of Walker's (22) studies have been published, and it is evident that his results also indicate rather clearly that different plant extracts affect differently the properties of a virus.

It may be suggested that the different experiments presented here should have been repeated many more times than was the case. The writer fully appreciates the value of repetition in experiments provided uncontrolled variables are not in excess of those which are controlled. It is believed that there are many uncontrolled variables in connection with the study of plant extracts and it is puzzling to know how best to control all of them.

The data presented seem to be very consistent in that they show no excessive irregularities. This in itself strengthens the results, and it is believed that more is to be gained from a study of the uncontrolled variables in plant extracts than would be gained from continued repetitions of the experiments made.

It is entirely possible that fluids from mosaic-free tobacco and cucumber plants may affect the properties of a virus somewhat differently than do the fluids from these plants when severely affected with mosaic. It is also fully recognized that in the experiments

reported here the virus concentration and type of diluting medium were unavoidably varied simultaneously. However, after taking these points into consideration, it appears that the data presented indicate rather clearly that the thermal inactivation of a virus and the time period over which it retains its potency at room temperature, are affected by the concentration of the virus, and also by the plant constituents in which the virus is contained.

It further appears from these data that the results of comparative studies on the properties of viruses causing mosaic on different species of plants can not be interpreted accurately until the viruses can be studied in nearly equal concentrations and in some standard fluid. It is possible to approach a uniform fluid by transmitting the viruses of different mosaics to some given host from which the virus-containing fluids can be expressed for detailed property studies. However, it is believed that the introduction of plant constituents associated with a virus into a given host may complicate such a method. This belief seems to have some support in the results obtained by Johnson (15) with the fluids of supposedly healthy potatoes. When the potato-plant fluids were introduced into healthy tobacco plants, an infectious disease resulted.

In the end, it would seem that we must look forward to the purification or isolation of the virus. Although several obstacles seem to be in the way of this accomplishment, it is believed that progress can be made in this direction. One of the chief obstacles has been that no adequate quantitative method was available for the estimation of the concentrations of viruses. However, it is believed that preliminary results, which are published in the accompanying paper (20), show that dilution and inoculation tests can be standardized to a point which will enable the detection of relatively small differences in concentration.

#### SUMMARY

The temperatures at which the virus of tobacco mosaic become inactivated depends on the concentration of the virus and on the nature of the plant extract.

Virus extract diluted one hundred times with water was inactivated in 10 minutes at a temperature between 82° and 84° C., whereas the same virus undiluted was inactivated at a temperature between 88° and 90°.

Fluid extract from healthy cucumber plants seems to have a more depressing effect on the potency of the virus than does an equal amount of water or tobacco fluid.

Virus diluted with water or tobacco and cucumber juices loses its potency more rapidly on standing than does undiluted virus extract. This is seemingly more marked when the dilutions are made with healthy cucumber extract.

The first reduction in the potency of diluted virus extract does not appear to be permanent in all cases. The nature of this reactivation may be the result of an adsorption phenomenon or of an actual increase in the number of virus particles which were not previously inactivated.

The properties of viruses from different species of plants can not be studied adequately in a comparative way until the viruses can be purified, brought to a fairly uniform concentration, and studied in reasonably uniform dispersing media.

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# QUANTITATIVE AND PURIFICATION METHODS IN VIRUS STUDIES<sup>1</sup>

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## INTRODUCTION

Studies on the viruses of the mosaic diseases indicate that much of the extraneous material must be removed from the virus extracts before it will be possible to carry out many important experiments in a satisfactory manner. In attempting purification, several methods are available, but, owing to the relatively small amount of study which has been directed toward this problem, little is known concerning the practicability of the methods.

On the assumption that the virus of mosaic is an independent organism, several workers have attempted to cultivate it, and although Olitsky (20)<sup>3</sup> claims success with the virus of tobacco and tomato, Mulvania (19) and Purdy (22) failed to verify his results. It is possible that under very special conditions which were not apparent to Olitsky, the virus will increase or become more potent in vitro. However, as pointed out in the accompanying paper (17), it seems to be unnecessary to assume that the virus is an independent organism in order to explain such results as were claimed by Olitsky. Although it is possible that the virus may be isolated by pure-culture methods, it appears that some attention should be directed toward physical and chemical methods of purification.

Several theories have been advanced regarding the nature of the mosaic virus, but none of our present methods seems to be adequate for testing these theories in a satisfactory manner. Several of the most basic methods which are required in the work will be applicable regardless of the nature of the virus, and it seems apparent that their best development is not dependent on the acceptance of any one of the present theories concerning the virus.

In attempting the purification of the virus by any method it is soon found that little progress can be made until a quantitative method is available. Such a method is necessary for determining the relative concentrations of virus in physical and chemical fractions as well as in culture solutions.

## QUANTITATIVE STUDIES

Although it is entirely possible that one might develop a chemical or a microscopical method which would be infallible for testing the concentration of the virus, it seems somewhat more hopeful at

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<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 37.

present to attempt the development of a method based on a biological assay, that is, the occurrence of disease symptoms.

The use of biological tests for quantitative determinations is not new. In the standardization of toxins and antitoxins, such methods have long been in use. Ehrlich (6), in his classic studies in immunology, found that he could standardize the concentration of diphtheria toxin and antitoxin by employing test animals, and his "minimal lethal dose," the minimum amount of toxin which will be sure to kill a normal guinea pig weighing 250 gm. in a period of four days, is still the basis for standardizing this toxin. Modifications of this method are also used for standardizing several bacterial toxins and vaccines.

It has been pointed out by Drummond and Watson (4) that the detection and estimation of vitamins in foods can best be accomplished through the use of biological tests in spite of the errors involved. In testing for the concentration of vitamins (7, 8, 25) the test animals are fed gradually increasing amounts of the test food, and the relative concentrations of the vitamin are determined on the basis of the behavior of the animals. Similar methods are also used in the standardization of certain drugs (2, 9, 21).

In view of the fact that several investigators have found that the mosaic viruses can be diluted to a considerable degree without completely losing their potency, it appeared to the writer that a system of dilution experiments might serve as the best basis for preliminary quantitative studies. The three following factors offered possible means for measuring relative concentrations of a virus: (1) The length of time elapsing between inoculation and the appearance of disease symptoms; (2) the degree or the intensity of the symptoms; and (3) the percentage of inoculated plants which develop mosaic within a given period.

In the studies on the scab of potato, the disease caused by *Actinomyces scabies* (11), and in those on the wheat foot-rots caused by *Helminthosporium sativum* (15), and *Ophiobolus graminis* (18), the writer observed that the number of infected individuals and the severity of the injury varied as the amount of inoculum used in the different experiments was varied. The literature on the mosaic diseases indicates that the number of plants which develop mosaic after inoculation also varies with the concentration of the virus (1, 3). However, it is now evident that the methods employed previously do not make it possible to detect differences in the concentration of virus in most extracts until dilutions ranging from 1,000 to 50,000 times are reached. Obviously, this is a disadvantage, and in view of the importance of detecting differences between more concentrated virus extracts and of the desirability of having some reasonably uniform quantitative method, it was necessary to carry out new experiments designed to furnish the required information.

Although Walker (24) considers that the dilution property is very irregular and unsatisfactory for purposes of comparing viruses from different species of plants, it became evident to the writer, after completing several experiments, that the dilution method can be used for quantitative work. However, success with the method depends on certain important factors and on methods which can be standardized to a reasonable degree.

(1) The virus must lend itself to study in the expressed fluids without losing its potency in a short time.

(2) The plant employed must be easily cultured under greenhouse conditions during as much of the year as possible.

(3) It must be highly and uniformly susceptible to the disease, and must develop definite symptoms.

(4) The plants used in a single experiment must be grown under the same conditions.

(5) Growing conditions for all experiments must be standardized as far as possible.

(6) Accidental infection through insect carriers, soil, contact with infected or infested materials, must be guarded against.

(7) The inoculation technic employed must lend itself to the greatest possible uniformity and certainty.

In considering the plant and the virus most suitable for the quantitative studies, it became evident that none of the cereals or other grass crops, or their mosaics, could be employed at the present time. While there are several dicotyledonous hosts which might have been used, none seemed so well adapted for the studies as the tobacco plant and the virus of its mosaic disease.

#### PLANT-CULTURE METHODS

##### SOIL

Early in these studies it was observed that cases of accidental mosaic sometimes occurred in experimental stock when there was no evidence of insect or accidental contact transmission through the above-ground parts. Since the writer (14, 16) had found that wheat mosaic may be transmitted through the soil, limited studies were made to determine whether tobacco mosaic might be transmitted in the same way.

The virus-free soil used in these experiments was obtained from an old wood lot near Madison, Wis. Previous tests had shown that the soil produced healthy plants. The seedlings were grown in this soil in flats. When sufficiently large they were transplanted to the experimental soils in 4-inch earthen pots which had been sterilized with steam.

TABLE 1.—*Number of plants showing tobacco mosaic in soil receiving different treatments*

Soil treatments	Number of plants tested	Number of mosaic plants
Experiment 1.		
Virus-free, control.....	20	0
Mosaic tissue and juice.....	19	14
Roots from mosaic plants.....	20	6
Experiment 2:		
Virus-free soil, control.....	24	0
Virus-free sand, control.....	24	0
Sand from beneath pots of mosaic plants.....	23	0
Virus soil, control.....	23	4
Virus soil+formaldehyde.....	24	0
Virus soil+uspulun (5 gm.).....	24	4
Virus soil+uspulun (10 gm.).....	24	6

In experiment 1 a series of seedlings was planted in virus-free soil; a series in soil in which mosaic-affected tobacco had recently grown, and which still contained the fresh roots of these plants; and a series in virus-free soil to which had been added, a few days previous to transplanting, juice and pulp from mosaic-affected tobacco plants. The results of experiment 1 (Table 1) indicate that infection does occur from the soil in a fairly large number of plants.

Experiment 2 was conducted to obtain some idea as to methods of controlling infection from the soil, and to determine whether potted plants would become diseased if placed on bench or floor sand on which potted mosaic plants had previously grown. The virus-free soil was of the same lot as that employed in experiment 1. The virus-infested soil was obtained from pots in which mosaic-affected plants had been growing. The fresh roots were present in the soil. The supposedly infested sand was obtained from a greenhouse bench on which mosaic-affected plants had been growing in pots immediately previous to the removal of the sand. The results of experiment 2 (Table 1) likewise show that infection may originate from the soil.

Johnson (10) has shown that tobacco does not grow well in many soils which have been sterilized with steam just before the introduction of the seedlings, and the writer's experience bears out this observation. Accordingly, chemical disinfectants were used instead of steam. As the writer had found previously that wheat mosaic can be controlled effectively when virus-infested soil is treated with a solution of 1 part fresh 40 per cent formaldehyde solution in 49 parts water, this treatment was employed. In addition, uspulun was applied in two concentrations, 5 gm. to a liter of water and 10 gm. to a liter of water. In all cases the infested soil was made into a stiff mud by the addition of the disinfectants, and by water in the case of the soil control. These soils were held in covered containers for 30 hours, and were then spread in layers and dried until no fumes of formaldehyde were noticeable from those treated with that chemical. They were then potted and the seedlings were transplanted into them.

Virus-transmitting insects which feed on the above-ground portions of the plants were guarded against. Very few insects were present outdoors during the period of these experiments and none were noted in the greenhouse in which the experiments were conducted. The uninoculated control plants were kept close to those planted in infested soil, and as they remained free from mosaic it is only reasonable to consider that the mosaic which occurred in the remaining plants had its origin from virus present in the soil.

From these preliminary tests it appears that soil treatments with freshly manufactured formaldehyde prevent tobacco-mosaic infection from the soil as effectively as they do wheat-mosaic infections from the soil, and that this disinfectant is more effective than uspulun in the concentrations employed.

From the one experiment it appears that bench sand may not be a serious source of accidental infection. This result is in harmony with field observations (16) in which the writer has found that wheat mosaic does not persist for as long a period in the sandy soil of the Illinois River region as it does in the heavy silt soil in the Mississippi River region near Granite City, Ill.

As a result of the experiments cited, every precaution was taken in the greenhouse studies to prevent accidental infection from the soil. If virus-free soil could not be obtained readily, other soil was disinfected with fresh formaldehyde. In all cases pots were sterilized with steam. Although it is possible that virus which gets into the sand under pots may in some cases cause accidental infection, it seems likely that most of this is washed deep enough to get below the few roots which may come through the pots in experiments of comparatively short duration.

If pots are placed in beds in the center of a house, it is well to set them on a layer of fine gravel several inches deep. By thoroughly wetting the gravel daily, and by disinfecting it several times a year, the possibility of accidental infection of young stock seems to be reduced to a minimum.

Since all possible infection from the soil and from other agencies has been guarded against, the number of accidental cases of mosaic has been noticeably reduced. In fact, accidental infection is so rare that it causes practically no concern.

#### PLANT STOCK

In the early experiments several varieties of tobacco were used. However, as the work developed all of the studies were confined to one strain of Connecticut Havana. The material from which the seed was obtained was kindly supplied by James Johnson. The seed used subsequently was derived from a single plant of this lot, growing in the field. In order to guard against possible cross-pollination, the plant was bagged before the flower buds opened. In all subsequent seed propagation the same precautions were taken. The seed from different plants was never mixed. It is believed that by following these methods all irregularities in infection due to genetic causes in the host were reduced to a minimum. In the winter the temperature in the growing house was kept as near 75° to 80° F. as possible. In the summer, temperatures fluctuated with the weather.

In all cases single plants were grown in 4-inch earthen pots. From 5 to 30 plants were inoculated with each sample of diluted virus extract, and all sets of plants inoculated with a given dilution were uniform in size and age. The plants were inoculated just before or during the period of their greatest vegetative development. In all of the earlier experiments healthy control plants were carried along with the inoculated plants, and in no case did mosaic occur in any of these during the first two weeks of an experiment. All experiments were discontinued at the end of this period.

#### INOCULATION TECHNIC

At the outset of the work it was found necessary to develop a method of inoculation by which it would be possible to obtain 100 per cent infection when the plants were inoculated with fresh, undiluted virus extract. After some experimenting, it was found that the percentage of mosaic plants resulting from inoculation with highly diluted extract increases as the amount of inoculum introduced into the plant is increased. To compare the results on a quantitative basis, it therefore becomes necessary to introduce as nearly as possible the same amount of diluted and undiluted fluid

into each plant in a given experiment. Owing to the nature of the vascular elements in tobacco, it is difficult, if not impossible, to inject a definite and constant quantity of fluid into the vascular system and have it all remain there. When a small hypodermic needle is inserted into the tissues practically all of the fluid injected comes out through the wound made by the needle.

After employing several methods of inoculation, the following was found both expedient and reasonably consistent in its results. A uniform amount of absorbent cotton lint was wrapped on the point of a stout, polished, steel needle. To this was applied several drops (one-eighth c. c.) of infectious fluid by means of a finely drawn glass pipette, or else the lint-tipped needle was dipped directly into the fluid. Either method gave satisfactory results. The saturated cotton was applied to the axil of a leaf near the base of the plant. The cotton was pushed down into the vascular region of the stem and petiole by means of the needle. The stem, petiole, and ligule were pricked and scratched at several points through the fluid which remained in the axil. Each inoculation needle was disinfected in 95 per cent alcohol, rinsed in water, and wiped before it was used to inoculate another plant. By using several needles the disinfection process caused no great inconvenience. Early in the work the operator's hands were washed in 95 per cent alcohol and wiped dry before an uninoculated plant was touched, but as the work progressed this operation seemed unnecessary, and it was employed only when proceeding from one lot of virus extract to another, or when it was necessary to handle some object which might be contaminated with a virus. Figure 1 illustrates the inoculation method.

Since using this method the writer has never failed to obtain 100 per cent infection in the many hundreds of plants inoculated with undiluted, fresh virus extract. Only in rare cases has he failed to obtain 100 per cent mosaic in plants inoculated with fresh extracts diluted 1,000 times, and in these cases there was evidence that the concentration of the virus was unusually low in the plants selected for the inoculum.

#### EXPERIMENTS

In the first experiments the writer inoculated five plants each of several series with lots of undiluted virus or with virus diluted 1,000, 10,000, 50,000, and 100,000 times in sterile distilled water. It was soon observed that the type of mosaic symptoms occurring on the plants inoculated with the virus in different concentrations varied so little, and these variations were so inconsistent or so difficult to measure accurately, that this factor could not be used to advantage in quantitative studies. In some plants a very mild type of mottling occurred. Several such plants were kept after closing an experiment, and in all cases they developed typical mottling as they continued to grow.

Although the time elapsing from inoculation to the appearance of mosaic was a little shorter for plants inoculated with the higher concentrations of virus, there was not sufficient regularity with respect to this manifestation to render it of any great value in quantitative studies. The most striking and consistent variable was the number of plants which developed symptoms within 4 to 15 days after inocula-

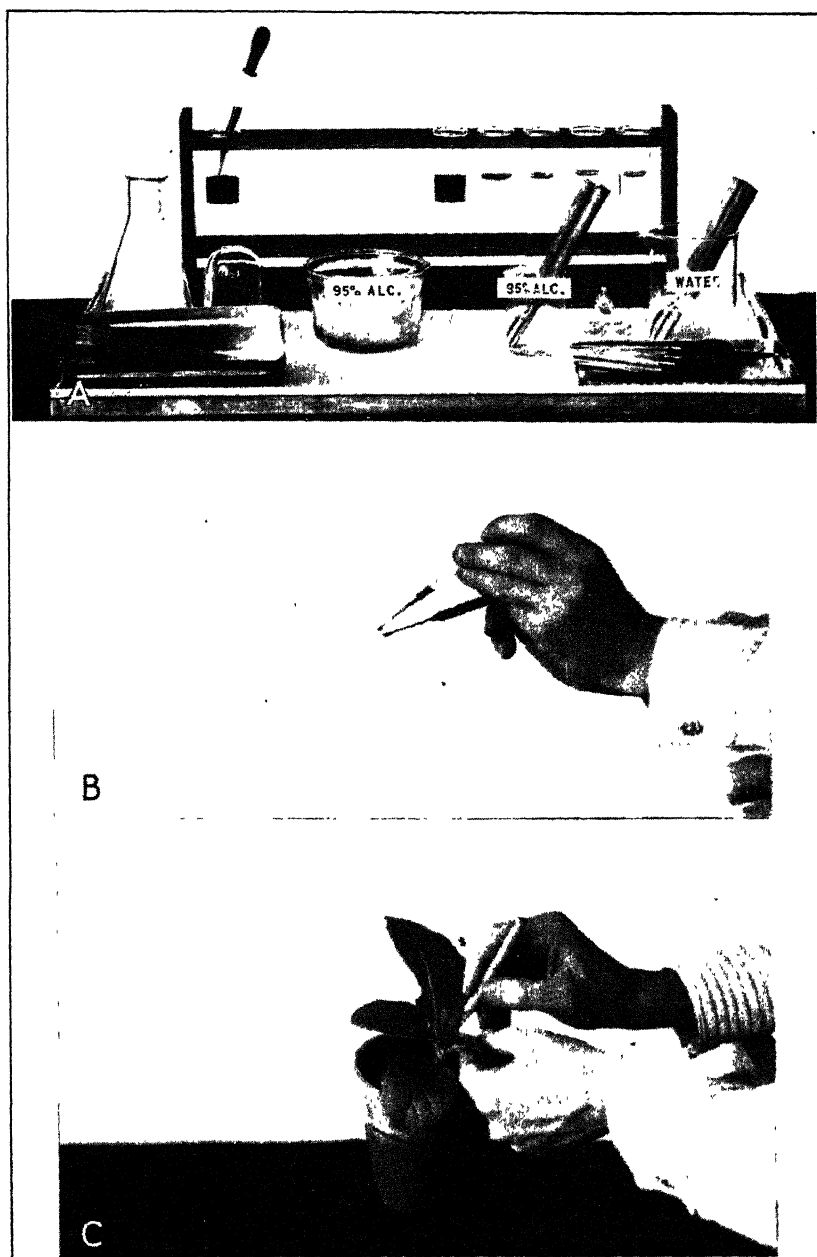


FIG. 1.—Materials and method used in inoculating the test plants. A, arrangement of the dilution samples and other items used for inoculating; B, method used for applying inoculum to the cotton on the tip of the needle (equally good results have been obtained by dipping the cotton in the extract contained in the vials, provided the fragments of cotton are of approximately the same quantity and compactness); C, method used in pushing the cotton and inoculum into the tissues at the juncture of the stem and the leaf petiole.

tion. As this relationship offered promising possibilities for measurement, other similar experiments were carried out. In some cases other dilutions were employed, but in general it was found that the series given above produced good results.

The time elapsing from inoculation to the first appearance of leaf mottling varies under different conditions. The period may be as short as four days when light, temperature, and nutrition are at their optima for rapid vegetative development. Only in rare cases has the time reached 12 days, and never in the writer's experiments has it exceeded 17 days. The time required for all infected plants to develop mosaic has varied from one to nine days. During periods of cloudy weather it requires a relatively long time for symptoms to appear in all infected plants in an experiment. When growing conditions are ideal this time is usually limited to three or four days.

In the course of the experiments precautions were taken for detecting accidental infection. Since all of the inoculated plants in a given experiment do not develop mosaic simultaneously, it is not always possible to detect accidental infections on the basis of the time required for the disease to develop. The writer now makes a practice of marking a young leaf on each plant by puncturing the leaf with the inoculation needle. By this method it has been possible to determine the occurrence of mosaic on leaves of different ages on many plants. Data from over 500 plants show that no definite mosaic occurred on leaves which were over 30 mm. long at the time of inoculation. Four per cent of the plants showed mosaic on leaves which were 25 to 30 mm. long, and 95 per cent showed mosaic on leaves which were 20 to 25 mm. long, and 100 per cent showed mosaic on leaves which were under 20 mm. long at the time the plants were inoculated. When mosaic occurs on leaves which are much over 30 mm. long at the time of inoculation, and the time required for mosaic to develop is unusually short, it is considered that the plant was probably infected previous to the experimental inoculation. When mosaic does not occur on leaves which were 15 to 20 mm. long at the time of inoculation and the symptoms are late in developing on younger leaves, it is considered likely that the plant became infected accidentally after the experimental inoculations were made. It was found that the mottling was very slight on many of the leaves which were 20 to 30 mm. long when the plants were inoculated. Although the data presented serve well for the conditions under which the writer has worked, it is possible that they may not serve for all conditions of growth.

A total of 35 dilution-inoculation experiments have been conducted with fresh virus extract obtained by pressing the juice from finely chopped, diseased plants. In all experiments infection occurred in 100 per cent of the plants inoculated with fresh undiluted virus. In 31 experiments infection occurred in all plants inoculated with fresh virus extract diluted 1,000 times. In six experiments 100 per cent infection occurred in all plants inoculated with dilutions of virus up to and including 10,000 times, and in one case infection occurred on all plants inoculated with all dilutions up to and including 50,000 times.

Owing to growth irregularities which occurred in seven of the experiments, the data from these were not averaged with those of the remaining 28 experiments.

The mean data obtained from the 28 experiments are given in Table 2 and are shown graphically by the dilution-infection curve in Figure 2.

In the case of the 100 per cent mosaic datum in Table 2 the dilution datum of 5,392 times represents the mean of the highest dilutions of the virus which produced mosaic in 100 per cent of the inoculated plants. The mean dilution data were determined for the lower percentages of mosaic by averaging the dilutions at which these percentages occurred. When these data are plotted on logarithmic paper the resulting curve tends to be of the logarithmic type. The datum point for 20 per cent infection is a little low, however, in comparison with the other points.

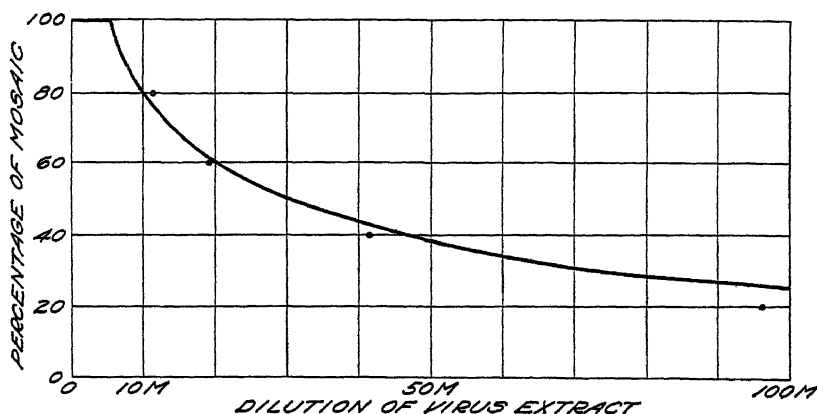


FIG. 2.—Dilution-infection curve based on the logarithmic curve of the mean data shown in Table 2. The curve is based on the average dilutions of virus at which the several percentages of mosaic occurred (Table 2)

The curve in Figure 2 was constructed on the basis of the logarithmic curve after it had been straightened. This seemed entirely allowable since the data conformed so closely to a straight line. For the present the curve in Figure 2 is being employed as a basis for the comparison of virus concentrations, and a unit of measure designated the "virus-unit" is proposed. This unit is defined as the concentration of virus in an extract which, on the average, is just capable of producing mosaic in 100 per cent of the plants inoculated according to the methods described. This concentration is designated the critical concentration. On this basis, the curve represents a concentration approximating 5,000 virus-units (5,000 V. U.) for the mean environmental conditions surrounding all of the experiments, since the mean critical concentration was reached at a dilution approximating 5,000 times.

TABLE 2.—Means of all dilutions of virus extracts which produced mosaic in 100 per cent and in lower percentages of the plants inoculated

Mean dilution	Percentage of mosaic
5,392	100
11,392	80
19,640	60
40,678	40
94,964	20

Figure 3 illustrates the curve in Figure 2 after it has been reduced to a single and fractional virus-unit basis. This was accomplished by dividing all of the dilution intervals given in Figure 2 by 5,000, the approximate mean critical concentration represented by Figure 2. Owing to the magnitude of this dilution, it seemed fair to neglect the fractional thousand and simplify the computations at this point. The figures on the horizontal axis of the curve in Figure 3 represent dilutions of a given volume of extract containing 1 virus-unit. The reciprocals of these dilutions represent fractions of a virus-unit. For example, at position 5 the extract has been made to five times its original volume, and, consequently, the concentration of virus in the resulting dilution equals one-fifth of a virus-unit ( $\frac{1}{5}$  v.-u.).

This curve can be rather accurately expressed by the equation

$$y = 130x^{-0.27} - 30, \text{ when}$$

$y$  = the percentage of mosaic-diseased plants obtained in a dilution test and

$x$  = the dilution of one virus-unit.

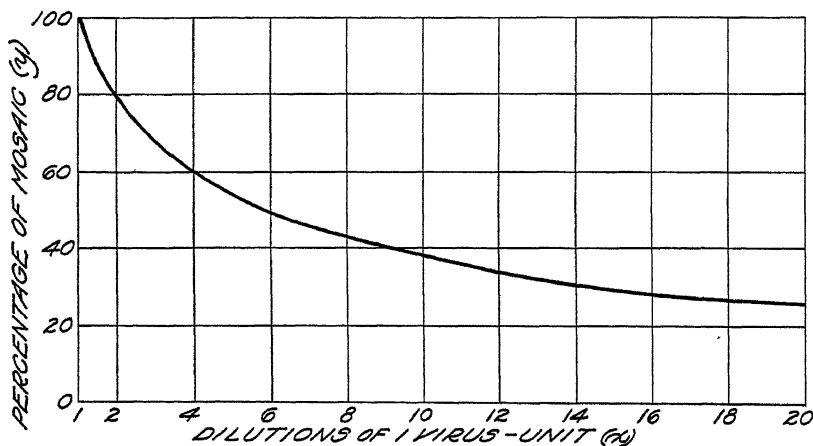


FIG. 3.—The dilution-infection curve illustrated in Figure 2 arranged on a single virus-unit (1 v.-u.) and fractional virus-unit basis. This arrangement was accomplished by dividing the dilutions (abscissae in fig. 2) by 5,000, the approximate mean critical concentration. Reciprocals of the dilution shown in this curve equal fractions of a virus-unit (1=original volume, i. e., 1 virus-unit; 2=double the original volume, i. e.,  $\frac{1}{2}$  virus-unit, etc.). This curve is used in this paper as the standard for comparing virus concentrations.

Whether this equation will hold with virus in all types of extracts is not known. It appears that it is influenced by environmental factors which influence the growth of the plant, but further study is required before these relationships will be understood. For the present it appears that the equation offers a reasonable basis for procedure, as it represents data obtained under all seasonal conditions in a greenhouse. However, it is emphasized that this position is only tentative. The summer season of 1925 at Madison was unusually cool and the sky was overcast for a greater number of days than usual. Observations at the Arlington Experiment Farm, Rosslyn, Va., also indicate that geographical locations are likely to affect the type of a dilution-infection curve to some extent. If this effect should prove to be great, it will be necessary to determine the type

of curve for the particular location where quantitative tests are conducted and to standardize all growth conditions as far as possible.

The question naturally arises as to why the number of mosaic-affected plants decreases gradually rather than abruptly as the virus is diluted beyond its critical concentration. At the present time it seems reasonable to explain this relationship on a chance basis. Regardless of the nature of the virus, its particles become more widely separated as the extract is diluted, and, accordingly, the chance that the required number of particles will be so placed in the plant as to produce mosaic becomes less as the dilutions increase. If the virus particles are adsorbed on colloidal or on larger particles in the extract, it seems probable that any change which may cause a release of the virus particles will increase the chances for producing mosaic.

The dilution-infection curve indicates that there is a marked acceleration in activity on the part of the virus after the dilution passes beyond the point of the critical concentration. Although these curves resemble a curve representing the actual reduction in concentration of any known substance which is being diluted, it is evident that the physiological activity of the virus as measured by mosaic symptoms is in excess proportion to the actual relative concentration of the virus in the diluted samples of extract. This accelerating effect may be due to conditions arising in the dilution samples or to conditions in the test plants. There is some evidence that the dilution-infection curve may rise slightly when the dilution of the virus extract is carried to 200,000 to 400,000 times, but this point can be tested in a more satisfactory manner after the completion of other studies.

The dilution-inoculation method may be applied in several ways, depending on the nature of the problem under investigation. When the investigator is not familiar with a virus extract it is very difficult to select at random those dilutions which will contain the critical concentration of the virus. In this case it is best to make several dilutions of the extract and to inoculate five or more plants with each. For this work the writer usually uses the extract undiluted and diluted 1,000, 10,000, 50,000, and 100,000 times in sterile distilled water. Further tests can then be made in the region near the critical concentration by employing several dilutions which may enable a more accurate location of this concentration.

The tests also may be carried out in the region between the critical concentration and the point of 40 per cent infection. When this method is employed the writer uses but one or two dilutions of the virus extract and from 10 to 30 test plants for each dilution sample.

After having determined the percentage of mosaic-diseased plants produced by an unknown diluted extract ( $y$  in the equation for the infection curve), the virus-unit concentration is computed according to the following equation.

$$V. U. = \frac{x'}{x} \text{ when}$$

$x'$  = dilution of unknown extract  
 $x$  = dilution of 1 virus-unit.

Example: If the experiment shows that an unknown extract diluted 10,000 times produces mosaic in 60 per cent of the plants inoculated, then  $x' = 10,000$  and  $y = 60$ . From the plotted curve

(fig. 3) it is then found that when  $y = 60$ , one V. U. has been diluted four times, or  $x = 4$ .

By carrying out the computation it is found that the

$$\text{V. U.} = \frac{10,000}{4} = 2,500,$$

that is, the virus concentration of the unknown sample approximates 2,500 virus-units for one-eighth c. c. of extract, the approximate volume used to inoculate each plant.

When several dilutions of the same virus extract are tested simultaneously the writer computes the virus-units for each dilution test beyond the point of 100 per cent infection and not including 0 per cent infections at the end of the series. All of the virus-unit determinations are then averaged, and this average is taken as the approximate virus concentration of one-eighth c. c. of the extract being tested.

As dilution-infection curves have approached 20 per cent to 0 per cent infection, experimental variation seemed to increase, and it has not been uncommon for infection to reach 0 at a given dilution and then rise a little at the next higher dilution. In this case the 0 per cent infection was averaged with the virus-unit calculations for the remaining dilutions. Sometimes the curve drops and rises again with the higher percentages of infection, especially when the dilution steps are close together. However, this has occurred in but few cases in which the populations were small.

In some cases, as in the experiments cited in Tables 4 and 5, the writer did not select dilutions sufficiently low to give the best infection data. In Table 4 it will be noted that in certain cases no infection occurred in any of the plants inoculated with extract diluted above two times. In such cases it is not possible to compute the virus-unit concentration of the fractions. However, from the data in Table 4 we know that the filtrate fractions did contain virus, because mosaic occurred in 100 per cent of the plants which were inoculated with extract diluted to twice its original volume. From the data in Table 5 there is no way of determining if virus was present in the filtrate fraction. As it is certain that these filtrates contained less virus than the unfiltered extracts, it is possible to determine the approximate highest value for the virus-units in them. In this case the writer assumes that if one more test plant had been inoculated with the filtrates diluted 1,000 and 100 times, respectively, it might have in each case developed mosaic. Thus, 24 virus-units in Table 4 and 1.3 virus-units in Table 5 represent the highest possible values for the fractions, and it seems likely that the values were actually less than these.

From the experiments carried out thus far, it is believed that the most accurate results can be obtained by determining the position of the critical concentration or by basing the virus concentrations on infection percentages which are close to the critical concentration. However, as stated previously, it is rather difficult, in most cases, to estimate the position of the critical concentration for purposes of selecting the dilution intervals which will yield data in the desired region of the curve. As a result, most of the writer's data include all or nearly all of the infection range of the dilution-infection curve.

When more is known of the relative influence of the growth factors on the concentration of virus in plants it will be easier to estimate the dilutions which will yield the most accurate data.

It may be suggested that, owing to the variables which are not under control, there is no justification for endeavoring to use a virus-unit system. The writer took this position for some time, and expressed the experimental results as percentages of the total number of plants inoculated. However, it was soon found that this method gave no idea as to numerical proportions between concentrations. For example, if a given fraction produced mosaic in 80 per cent of the plants inoculated, and the same dilution of another fraction caused mosaic in 40 per cent of the plants, it could not be concluded that the first fraction had double the virus concentration of the second fraction. This is evident from the type of infection curves shown in Figures 2 and 3. As a matter of fact, the first fraction is about five times more concentrated than the second. It seemed advisable, therefore, to devise a method which would express proportions with greater exactness, and, while several possibilities presented themselves, the virus-unit method appeared reasonably satisfactory for the present.

From the results obtained thus far, it is evident that experiments of a similar nature should be repeated several times. Large populations of test plants should be employed when possible. To this end, the number of dilutions should be reduced as much as possible in order to facilitate the use of larger numbers of plants for a dilution test. In this way experimental variation may be reduced to the minimum.

## PURIFICATION STUDIES

### FILTRATION

Of the physical methods of separation, filtration probably has been used more generally than any other for purifying many biological compounds. In studies on the virus of mosaic it has been demonstrated many times that the virus passes certain filters and, consequently, the filtered virus is partially purified through the removal of the larger suspended and colloidal materials which were present in the original extract. It has long been known that ordinary charcoal and sand filters remove more of the impurities from water after a considerable amount of debris or slime has collected in the filters than they did before. This relationship is known also to hold generally in commercial filtration processes, and it has been recognized by those who produce various biological products, such as serums, vaccines, and antitoxins, that filter slime may itself be a good filter, depending on its nature (13).

In the ordinary filtration procedures with paper filters it is evident that a filtrate of extract from freshly collected plant leaves becomes decidedly clearer as filtration proceeds. A simple experiment conducted with soft filter paper, an ordinary ribbed, glass funnel, and 400 c. c. of freshly expressed juice from tobacco leaves showed that the first 10 c. c. of filtrate contained sufficient chlorophyll to render it almost as green as the unfiltered juice. The second 10 c. c. contained very little chlorophyll, but there was much brown-colored material in suspension; so much, in fact, that light from a 100-watt mazda lamp with a concentrated filament would not pass through the filtrate when in a test tube 16 mm. in diameter. In the case of the

fourth 10-c. c. sample of filtrate, the light passed through slightly, and in each succeeding sample it passed more readily, until in the fourteenth 10-c. c. sample, the filtrate was very clear, and the light shone through with great brilliance. When all of the samples of filtrate had been heated in a steamer long enough to bring about coagulation of part of the proteins, it was found that the amount of coagulum gradually decreased from the first to the fourteenth sample.

With this evidence in mind, it appeared reasonable to believe that the slime which collects on the surface of biological filter candles does act as a filter, thus rendering it difficult to determine the actual filtration capacity of a given candle for the virus of mosaic.

To test this point with a virus, the writer set up an experiment in which ordinary, soft-paper filters were employed in Büchner funnels 4 inches in diameter. Fresh, undiluted juice from mosaic-affected tobacco plants was heated at 65° C. for 70 minutes in order to coagulate the suspended material and a portion of the heat-coagulable proteins. This heating does not seem to impair the potency of the virus, and the resulting coagulum makes possible the use of paper filters for clarifying small volumes of fluid.

After the coagulation process, two 15 c. c. samples of the fluid were put in two Büchner funnels and the filtrates were obtained under a vacuum of 30 inches of mercury. The resulting filtrates were very clear. One of these, and also its filter slime, were tested for virus concentration. The other filtrate was discarded, but its slime was carefully washed on the filter with 150 c. c. of cold, distilled water, after which it was made up to the original volume (15 c. c.) and tested for virus concentration. The results of these experiments are shown in Table 3.

TABLE 3.—Virus concentrations in unfiltered fresh virus extract; in unfiltered fresh extract, heated at 65° C. for 70 minutes and filtered through soft filter paper; and in filter-slime precipitate, unwashed and washed

Materials	Percentage of mosaic at dilutions of a—							Average virus-units
	0	100	1,000	5,000	10,000	50,000	100,000	
Unfiltered extract.....	100	100	100	80	60	20	0	2,222
Filtrate.....	100	100	40	0	0	0	0	111
Precipitate.....	100	100	100	80	60	20	0	2,222
Washed precipitate.....	100	100	100	60	0	20	0	988

a Five plants inoculated with each dilution.

From these data it is evident that filter slime, under certain circumstances at least, does remove a considerable quantity of the virus. The results from the washed precipitate seem to indicate that a considerable portion of the virus was held by the slime in a physical rather than a chemical manner.

Further tests made with filtrates from fresh, unheated juices, were low in virus concentration as shown in Tables 4, 5, and 6. In Table 6 it will be seen that the removal of part of the slime by means of the centrifuge previous to filtration seemed to enable a greater quantity of virus to pass the filter than when all of the slime was present.

Further tests on the filtration of substances have shown that the presence of filter slime offers considerable difficulty, and it now appears that, under certain conditions, the colloidal and other suspended materials which make up the slime are effective filters.

TABLE 4.—*Virus concentrations of unheated fresh virus extract, diluted with an equal quantity of distilled water; unfiltered and filtered*

Materials	Percentage of mosaic at dilutions of <sup>a</sup> —					Average virus-units
	2	1,000	10,000	50,000	100,000	
Unfiltered extract.....	100	100	60	40	0	4,027
Berkefeld V filtrate <sup>b</sup> .....	100	0	0	0	0	<sup>c</sup> 24
Berkefeld V filtrate <sup>b</sup> .....	100	0	0	0	0	<sup>c</sup> 24
Berkefeld W filtrate <sup>b</sup> .....	100	0	0	0	0	<sup>c</sup> 24

<sup>a</sup> Five plants were inoculated with each dilution.

<sup>b</sup> Two tests were made on the "V" filtrate and one test was made by passing the "V" filtrate through a "W" candle.

<sup>c</sup> This value is based on the possibility that one out of six plants inoculated with extract diluted 1,000 times might have developed mosaic. See explanation in text.

TABLE 5.—*Virus concentrations of unheated undiluted virus extract; unfiltered and filtered*

(The extract stood in the laboratory for five hours before the filtration was made)

Materials	Percentage of mosaic at dilutions of <sup>a</sup> —			Average virus-units
	100	1,000	10,000	
Unfiltered extract.....	100	100	90	7,692
Berkefeld V filtrate.....	0	0	0	<sup>b</sup> 1.3

<sup>a</sup> Ten plants were inoculated with each dilution.

<sup>b</sup> This value is based on the possibility that 1 out of 11 plants inoculated with extract diluted 100 times might have developed mosaic. See explanation in text.

TABLE 6.—*Virus concentration of fresh undiluted virus extract; of a portion of the same extract after it had been centrifuged for seven minutes at approximately 50,000 revolutions per minute, and of filtrates from fresh and from centrifuged extract*

Materials	Percentage of mosaic at dilutions of <sup>a</sup> —						Average virus-units
	0	500	1,000	10,000	50,000	100,000	
Fresh extract.....	100	-----	100	80	0	0	5,000
Centrifuged extract.....	100	-----	100	40	0	0	1,111
Filtrate, fresh extract <sup>b</sup> .....	100	20	20	0	0	-----	25
Filtrate, centrifuged extract <sup>b</sup> .....	100	60	80	20	0	-----	317

<sup>a</sup> Five plants were inoculated with each dilution.

<sup>b</sup> A Berkefeld V filter candle was used.

By using hemoglobin as a test substance, it was found in two experiments that approximately 18 and 50 per cent of the hemoglobin in 1 per cent water solutions passed a Berkefeld V filter ( $\frac{5}{8}$  by  $1\frac{1}{2}$  inches) at the rate of 10 c. c. in 45 and 15 seconds, respectively. The reduced concentration of hemoglobin in the first filtrate was due to extraneous material which collected on the filter and formed a slight

slime. This material was removed from the hemoglobin by means of a supercentrifuge before the second experiment was started. A 1 per cent hemoglobin solution was then prepared by mixing a 2 per cent water solution of hemoglobin with an equal quantity of fresh, undiluted, unfiltered tobacco fluid from very young plants.<sup>4</sup> This solution was filtered in the same filter candle referred to above, after it had been thoroughly cleaned. No visible hemoglobin passed with the filtrate. In fact, the presence of the hemoglobin in the unfiltered extract so improved the filtering properties of the slime deposit that about one-half of the lemon-yellow pigment in the fluid was removed when the filtrate came through the candle.

Three hours were required for 7 c. c. of this filtrate to pass the filter. By carefully washing the filter it was found that no visible hemoglobin got beyond the filter slime. This whole experiment was repeated and the same results were obtained.

Although the filtration experiments are preliminary, the results seem sufficiently consistent to warrant the general conclusion that filtrates are relatively low in virus concentration. The filtrate concentrations, as found in these experiments, ranged from about 20 to 5,900 times less than the concentrations of the unfiltered extracts. It is apparent that certain of the suspended materials in an extract constitute a source of error and difficulty in filtration studies, and it appears that unpurified virus can not be employed with certainty for studies on the determination of the size of the virus particles by means of filters (5).

This interpretation is borne out by results of colloid chemists who have studied particle sizes by means of filters. Even with purified colloids it is found that filters offer many difficulties, some of which may be reduced through the use of the supercentrifuge (23).

In some cases the variability of filtration of a given substance has been explained on the basis of differences in the electrical charge on the filter. This explanation is doubtless correct for substances which have been purified to a considerable extent. However, in the case of complex plant extracts and animal sera it is likely that the effect of an electrical charge is sometimes more indirect than direct. The physical arrangement of the suspended materials in plant extract can be greatly altered by changing the electrical charge. Thus, the arrangement of these materials (slime) on the filter will alter the porosity of the slime layer and thereby alter the passage of virus.

#### GRAVITATION AND SUPERCENTRIFUGATION

As the quantitative virus studies indicated that the filtration process offers several difficulties when it is desired to recover highly concentrated virus, it seemed desirable to explore the possibilities of using gravity and centrifugal force to effect the desired separations. By these methods it is possible to recover all fractions more completely than by the use of filters. Moreover these methods do not introduce the difficulties resulting from the slime layer which forms on filters.

A preliminary experiment was carried out with fluid from tobacco plants affected with mosaic. This fluid was allowed to stand in a flask in a refrigerator for 12 hours. At the end of this period the re-

<sup>4</sup> The filtered juice from these young tobacco plants was colored a faint lemon yellow, and small traces of hemoglobin could be detected in it quite readily.

sulting supernatant fluid was decanted into another container. At the end of another 12 hours a second supernatant fluid was evident in the container which held the original sediment. At this time samples of the first and second supernatant fluids and of the final sediment were tested for virus concentration. As indicated in Table 7, the greatest virus concentration occurred in the supernatant fluid next above the sediment.

TABLE 7.—*Virus concentrations of three gravity fractions of virus extract which had been diluted with an equal volume of distilled water and allowed to stand in a refrigerator for 12 hours*

Fractions	Percentage of mosaic at dilutions of <sup>a</sup> —						Average virus-units
	2	2,000	10,000	20,000	100,000	200,000	
1 (upper).....	100	100	100	80	40	20	9,259
2 (intermediate).....	100	100	100	80	80	60	36,666
3 (bottom).....	100	100	40	60	0	0	3,055

<sup>a</sup> Five plants were inoculated with each dilution.

The results of this experiment indicated that much of the extraneous material in fluids might be removed by centrifugation without reducing the virus concentrations as rapidly as by filtration. Preliminary tests with ordinary types of test-tube centrifuges soon indicated that the speeds were too low to effect good separations of fresh fluids. It was found that separations were much more easily made in fluids which had been heated at 65° C. or allowed to stand in the laboratory long enough to effect coagulation of a portion of the suspended materials. However, it was soon apparent that the juices from different lots of plants of the same species varied considerably. Some would not show coagulation on heating at 65° or on standing at room temperature for long periods. For this reason, experiments were carried out with high-speed centrifuges.

The Sharpless supercentrifuge was first employed, and it was found that much of the suspended material in the most refractory extracts could be removed in a few minutes when the fluids were rotated at 40,000 revolutions per minute. The laboratory model of this centrifuge offers several possibilities, but it was found to be too large for studying small volumes of fluid.

Search for a centrifuge revealed one which, after some special adaptations, served admirably for these studies. This machine is an outgrowth of several centrifuges developed by E. A. Birge and C. Juday for their studies on the Wisconsin lake plankton. It had been described by Juday (12) and is again described in this paper in order that certain modifications may be noted (figs. 4 and 5).

The machine consists of a Dumore No. 3 multispeed grinding motor and spindle mounted vertically on a right-angle cast-iron base. The centrifuge cup is screwed on the upper end of the spindle.

For centrifuging a continuous flow of fluid the cup is mounted in a brass housing on the top of the spindle casting. For the study of small batches of fluid this housing is not necessary.

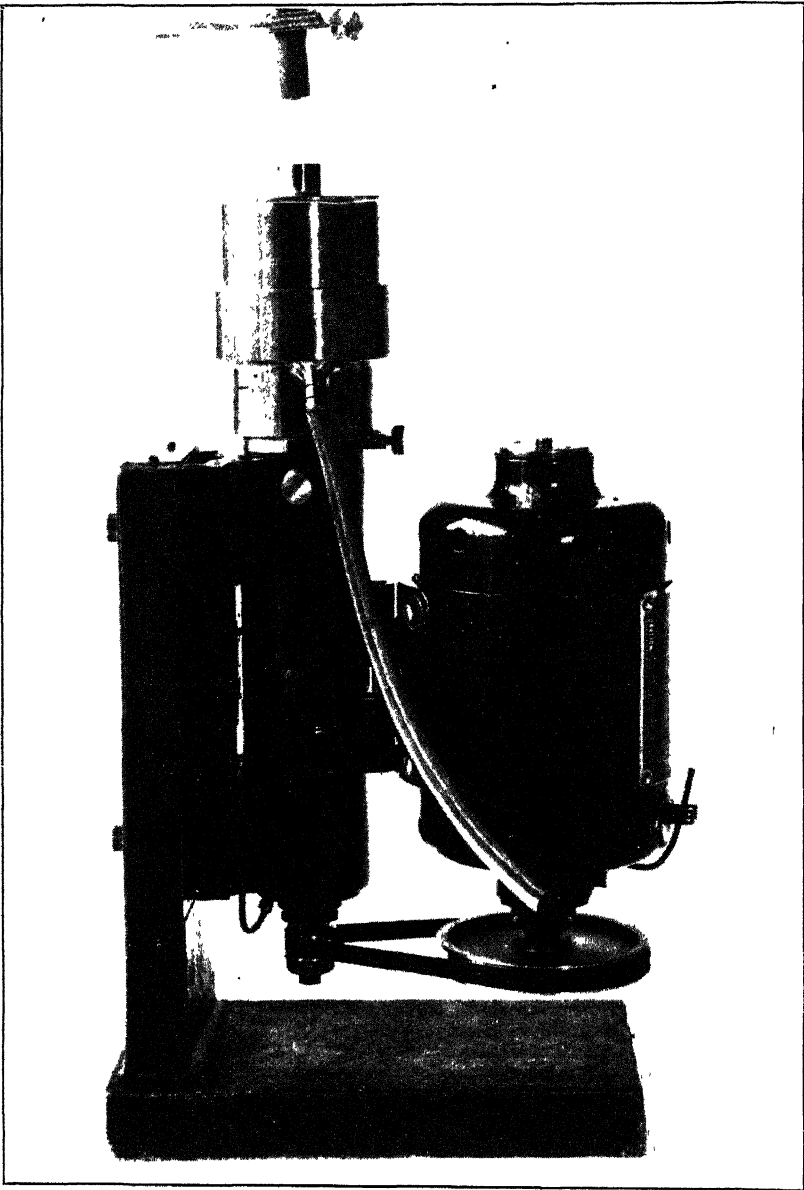


FIG. 4.—Supercentrifuge, constructed by attaching a high-speed motor and grinding spindle to a cast-iron upright. The centrifuge bowl is screwed on the top end of the spindle, which is inclosed in the housing and water jacket shown in Figure 5. As illustrated, the machine is arranged for centrifuging a continuous flow of fluid entering at the top. The solid material remains in the bowl and the liquid fraction runs out through the lateral rubber tube

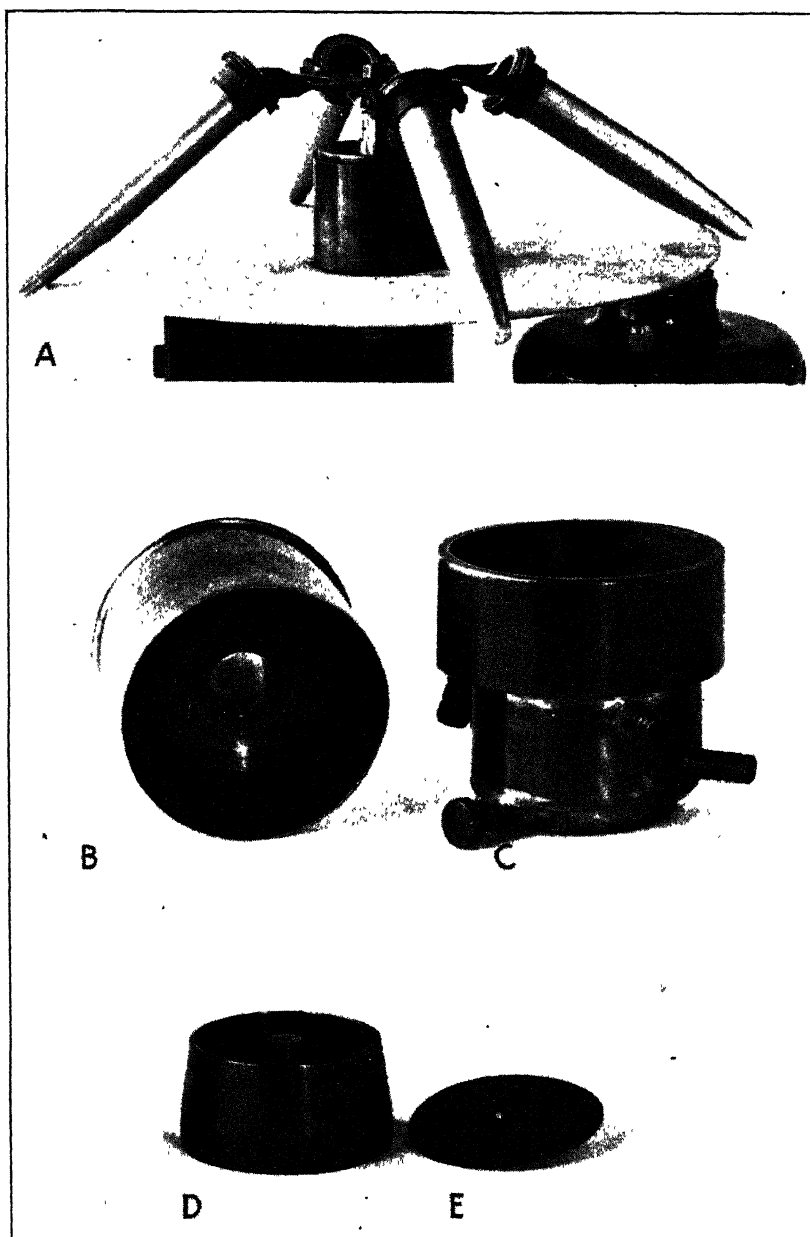


FIG. 5.—Parts employed in connection with the centrifuge shown in Figure 4: A, an ordinary sedimentation tube head which can be put on the spindle in place of the bowl; the metal disk prevents the shields from striking the motor and support; B, the housing cover, which is used when centrifuging a continuous flow of fluid; this cover fits into C, and its internal disk fits into the bowl D to prevent the liquid from flying out of the bowl before it has been subjected to centrifugal force; C, the housing which serves as a collecting head for the centrifuged fluid that comes over the upper edge of bowl D, and also as a water jacket for cooling the upper bearings of the spindle; D, the bowl; this rotates in B and C when a continuous flow of fluid is being centrifuged; E, a thin spring-brass cover which is screwed to the central post in cup D, when small quantities (4 to 8 c. c.) of fluid are centrifuged; in this case the housing cover B is not used

In most of the studies single small lots of fluid, ranging from 4 to 10 c. c. were employed. As the centrifuge is run at its maximum speed, approximately 50,000 revolutions per minute, it is obvious that a great amount of evaporation takes place in an open cup such as was employed by Juday. For this reason the writer now uses a bronze bowl 2 inches in diameter and 1 inch deep (inside dimensions). The walls form an acute angle of  $8^{\circ}$  with the bottom. By turning a small bead on the inside of the top edge the bowl holds approximately 8 c. c. of fluid when running at full speed. The central post in the bowl is slightly lower than the edges of the wall. A small hole in the top of this is tapped to take a small screw, which fastens a very thin, spring-brass cover over the bowl. This cover prevents evaporation and thus enables accurate tests to be made on the different fractions.

Earlier, a bowl with vertical sides was constructed, with a cover which screwed onto the walls of the bowl. After some experimenting, it was found almost impossible to obtain a sufficiently tight cover to prevent the loss of the fluid while it was being centrifuged, and as a consequence this construction had to be discarded.

In certain studies it was necessary to centrifuge for a rather long time, and frequently the machine was in use over long periods while several successive samples were being centrifuged for short intervals. Under these conditions the upper bearings of the spindle got very warm and the speed was sometimes reduced on this account. To eliminate this difficulty the writer designed a water jacket at the base of the bowl housing, as shown in Figure 5, C.

To oil the bearings small copper pipes were inserted in the oil holes at the bottom bearings of the motor and spindle. These were turned upward so that the oil might be introduced readily. It is estimated that this machine exerts a force almost 70,000 times greater than the force of gravity when rotating a bowl 2 inches in diameter at approximately 50,000 revolutions per minute.

In addition to the mechanical problems which arose, many new problems were encountered in connection with plant fluids and with the virus. For this reason the data presented can not be considered final. However, in general, they are so consistent that it seems worth while to present them in summarized form in order to throw further light on the virus and to show more clearly the value of the quantitative method which has been described.

In all of the experiments carried out the solid fractions were carefully removed in a volume of sterile distilled water equal in weight to the fluid fraction removed. Therefore, the virus concentrations in all fractions were compared on practically an equal-volume basis. The differences in the specific gravity of the fluids were so small that they are ignored in the present discussion.

Four experiments were conducted to determine the concentration of virus in the heavy chlorophyll-bearing material present in fresh extract from diseased plants. This fraction usually can be separated from 4 c. c. of fluid in about 15 seconds by centrifuging at full speed. From results of these experiments, shown in Table 8, it is evident that this first heavy fraction is very low in virus content as compared with the remaining fluid fraction.

TABLE 8.—*Virus concentrations in solid and fluid fractions from a fresh virus extract centrifuged in a closed bowl for one minute at approximately 50,000 revolutions per minute*

Experiment No.	Fraction	Percentage of mosaic at dilutions of a—					Average virus units
		0	1,000	10,000	50,000	100,000	
1.....	Control (un-centrifuged extract)	100	100	60	20	20	2,499
2.....	do.....	100	100	40	20	20	2,036
1.....	Solid.....	100	40	0	0	0	111
2.....	do.....	100	40	0	0	0	111
3.....	do.....	100	20	0	0	0	33
4.....	do.....	100	80	0	0	0	500
Mean.....							188
1.....	Fluid.....	100	100	40	20	20	2,036
2.....	do.....	100	100	20	20	0	1,000
3.....	do.....	100	100	80	60	20	6,944
4.....	do.....	100	100	80	20	20	3,333
Mean.....							3,328

\* Five plants were inoculated with each dilution.

An experiment was carried out to determine if centrifuging the extract for longer periods would cause the virus concentration in the solid fraction to increase. It was found that the concentration did increase in the solid fraction when the fluid was centrifuged for nine minutes, but the increase did not seem to be very great.

It was then decided to determine the effect of combining heat treatment with long centrifuging. The results of this experiment are given in Table 9. It will be seen that this combination makes possible the removal of a large percentage of the virus from the fluid fraction.

Eight additional experiments were conducted on this phase of the problem. The data are given in Table 10. In each of these experiments it was found that the virus concentration in the solid fraction from whole extracts which were centrifuged for short intervals (one-half to one minute) was low, and that the virus concentration in the fluid fraction was high. However, by heating the extracts at 65° C. until coagulation occurred and then centrifuging them for 5 to 10 minutes, it was possible to remove a large percentage of the virus from the fluid and concentrate it in the solid fractions.

In all of the centrifugation studies thus far conducted it appears that the virus concentration of the fluid fraction can be reduced most readily in extracts which have been heated at 65° C. for 10 to 15 minutes, or which have stood in the laboratory for several hours.

In general, it seems evident that the results of the gravitation and the centrifugation experiments agree in that the virus concentration is greatest in what might be termed the "intermediate" fraction. Whether the small amount of virus which remains in the fluid fraction after long centrifugation is the result of contamination from the solid fraction, or whether it represents a form which is in solution, is not known. When the centrifuge stops there is considerable washing of the solid fraction by the liquid fraction, and a portion of the virus which has been deposited may be taken up again by the fluid. The ultimate solution of this problem depends to some extent on further improvement of the apparatus.

TABLE 9.—*Virus concentrations in solid and fluid fractions from fresh virus extract centrifuged for 2 seconds; in solid and fluid fractions from virus extract which, after having stood for 4 hours at room temperature, was heated at 65° C. for 15 minutes and centrifuged for 10¼ minutes at approximately 50,000 revolutions per minute*

Fractions	Percentage of mosaic at dilutions of a—			Average virus-units
	100	1,000	10,000	
Solid fraction from fresh extract centrifuged for 2 seconds <sup>b</sup> .....	90	40	20	171
Fluid fraction from fresh extract centrifuged for 2 seconds.....	100	100	100	10,000
Solid fraction from 4-hour extract, heated at 65° C. for 15 minutes and centrifuged for 10¼ minutes <sup>c</sup> .....	100	100	80	5,000
Fluid fraction from 4-hour extract, heated at 65° C. for 15 min- and centrifuged for 10¼ minutes.....	90	50	10	128

<sup>a</sup> Ten plants were inoculated with each dilution.

<sup>b</sup> It was found that practically all of the chlorophyll-bearing material in this particular sample of extract was removed from the fluid by centrifuging for the unusually short time of 2 seconds at full speed.

<sup>c</sup> This fraction was obtained by centrifuging the sample in two operations, thus causing some loss of virus. However, it is believed that the difference between the concentrations of virus in the fluid from the sample centrifuged for 2 seconds and of that in the solid fraction from the sample centrifuged for 10¼ minutes is largely due to experimental variation which would have been reduced if a larger number of test plants had been used.

TABLE 10.—*Mean data from eight experiments, showing the virus concentration in solid and fluid fractions from extracts centrifuged for short periods (one-half to 1 minute) without heating, and for long periods (5 to 10 minutes) after heating at 65° C.*

Time of centrifuging	Fraction	Mean percentage of mosaic at dilutions of—							Average virus-units
		0	100	1,000	10,000	25,000	50,000	100,000	
Short time.....	{Fluid.....	100	100	90	50	30	20	20	1,842
	{Solid.....	100	70	43	10	6.6	0	0	142
Long time.....	{Fluid.....	100	73.3	48	32	6.6	0	0	307
	{Solid.....	100	100	90	50	40	0	0	1,755

## STUDIES ON THE VARIABILITY OF VIRUS EXTRACTS

The quantitative method has been applied to several virus problems other than those cited, but there are fewer data on these. However, it may be stated that evidence now available indicates that virus concentrations in expressed extracts are influenced considerably by the method of extraction. In one experiment the extract from fresh mosaic-affected tobacco leaves, passed through the fine cutter of a food-chopper, had a virus concentration of 6,332 virus-units. When the press cake or tissue from which this extract was removed was ground with quartz sand in a quantity of water equal to the quantity of extract removed the resulting expressed extract contained 5,479 virus-units.

In a second similar experiment it was found that the first extract contained 6,667 virus-units, and the second extract, obtained after grinding the press cake with sand, contained virus amounting to 3,333 virus-units. A hand press was used in all of the experiments reported. Had a hydraulic press been available, it is probable that the virus concentrations of extracts from tissue passed through a food chopper would have been greatly increased. Doubtless the crushing of the cells with sand aids in increasing the yield of virus.

However, it appears that the presence of the sand in the press cake aids the virus in escaping from the press cake into the press fluid. Experiments on frozen tissue seem to bear out this point to some extent.

Virus concentration also varies in different parts of the same plant. In one experiment it was found that extract from the six uppermost leaves of a mosaic-mottled tobacco plant contained virus amounting to 3,072 virus-units. The extract from the stem to which these leaves were attached contained only 33 virus-units. In a second similar experiment the leaf extract contained 38,461 virus-units and the stem extract 3,255.

It has been found that the virus concentration is from two to four times greater in succulent plants than in older woody plants. The upper mottled leaves of a diseased plant contained about one-third more virus than the lower unmottled leaves of the same plant.

Extracts from the tobacco plant vary greatly in their ability to produce a marked coagulum and precipitate on standing at room temperature or on being heated. In some cases a marked coagulum will develop and settle out of expressed tobacco fluid when it has stood for two or three hours, whereas fluid from tobacco plants grown under different conditions may show no change to the eye after standing three or four days. Under certain conditions, a good coagulum will develop and settle out of freshly expressed tobacco juice when heated 5 or 10 minutes at 40° C. yet it is not uncommon to find tobacco extract which shows no apparent coagulation until the temperature reaches 90° or above. All gradations of coagulation have been observed between these temperatures. On further study with supercentrifuged extracts it was found that some of the protein and other substances do coagulate in all of these fluids when heated. However, under certain conditions this is masked by the chlorophyll-bearing material, which does not always coagulate and settle out at lower temperatures. In general it may be said that the extracts from tobacco plants grown in the summer coagulate more readily than do those from plants grown in the autumn, winter, or spring.

Juices from all plants of the same species do not filter alike. In some cases it is possible to obtain complete filtration of a given quantity of fresh extract in from 10 to 15 minutes, whereas an equal quantity of another lot of fluid similarly handled may require 3 to 4 hours to pass the same filter candle. In the centrifugation studies it was found that some fluids would clarify in 5 seconds, whereas others required almost 60 seconds to reach the same degree of clarification. Extracts from summer-grown tobacco plants seem to filter and clarify more readily than do those from plants grown in the autumn, winter, and spring. Extracts from old plants are less easily filtered and clarified in the centrifuge than are the extracts from younger plants. Several tests have shown that extracts from old plants do not produce a coagulum as readily on heating as do the extracts from young plants. The age of plants seems also to influence the rate of precipitation of a coagulum produced by heat or by standing at room temperature. The conditions that regulate the growth processes—as nutrition, temperature, soil, atmospheric moisture, and light—seem also to regulate the behavior of plant extracts in vitro. It would therefore appear that if uniform extracts are to be obtained

for use in experiments of the sort described, more attention must be given to the regulation of the growth conditions and to the selection of the plants employed.

In addition to the variables which are unavoidable in the culture of the plant there are others which are due to the methods employed in handling the extracts. In general it has been found that extracts filter and also clarify in the centrifuge more readily after they have stood in the laboratory for some time, been frozen, treated with certain chemicals, or after they have been heated at 65° C. for 5 to 10 minutes. It is apparent that anything which changes the physical or chemical state of the suspended material in an extract is likely to affect filtration and clarification.

### CONCLUSIONS

Experiments which are presented in this paper indicate that the virus of tobacco mosaic can be studied quantitatively through the use of dilution and inoculation methods. The method which is suggested has been tested in connection with the study of several problems, and the relative congruity of the virus concentrations in fractions obtained in a similar manner lends support to the method.

The accuracy of the method depends on several factors which are not easily controlled. In order to reduce experimental variation to a minimum, it is necessary to use large areas of greenhouse space for the many test plants. This makes it difficult to control temperatures as accurately as would be possible in small chambers. At the present time it is impossible to obtain even approximately uniform sunlight conditions for experiments carried on at different periods of the year. However, in spite of these variables, it seems evident that the method offers a basis for the further improvement of quantitative methods.

The preparation of virus in some standard form which may be employed as a control in quantitative tests of virus of unknown concentration should tend to simplify the present difficulty of growth variables which occur in experiments that are not conducted simultaneously. A similar procedure has made it possible to perfect the biological assay of certain drugs and antitoxins.

Data presented show that filtrates of virus extract tend to be low in virus concentration. These data indicate that the filter slime, which is composed of suspensoidal and colloidal materials contained in the extracts, removes a considerable amount of the virus. On this account it is impracticable to employ filters in certain stages of purifying a virus and in making studies on the size of the virus particle.

Tests carried out with a supercentrifuge show that the heavy suspended particles in a virus extract can be removed by centrifugal force without seriously reducing the concentration of the virus in the fluid fraction. A large proportion of the virus can then be removed from the fluid by heat coagulation and further centrifugation.

Preliminary experiments indicate that the concentration of the virus varies in plants grown under different conditions, in plants of different ages, and in different parts of the same plant. It was also found that the concentration of virus in an extract is influenced by the methods used in preparing the tissue and in making the extraction. The chemical and physical behavior of extracts also varies in plants

of different ages and in plants of the same age but which are grown under different conditions.

From the results presented, it is evident that the methods of culturing and selecting the plant material, and the methods employed in making and manipulating the virus extracts must be standardized just as far as possible to insure reasonably uniform results.

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# AGE OF POLLEN AND OTHER FACTORS AFFECTING MENDELIAN RATIOS IN MAIZE<sup>1</sup>

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## INTRODUCTION

Mendelian inheritance is based upon the assumption that the heritable elements or genes derived from the parents remain unchanged and are distributed equally to the gametes. Under normal conditions, therefore, the gametes of hybrids should fall into two equal classes with respect to any given character pair. That such is indeed the fact is attested by innumerable experiments. With some characters, it is true, orthodox ratios are not always found. Most of these cases, however, can be explained by a differential viability of the zygotes or other causes not associated with the ratios of the genes in the gametes.

The present thesis is that modification of the Mendelian ratios can occur in the gametes. The genes here dealt with are the *C c* pair involved in the production of aleurone color in the seeds of maize, and the *Wx wx* pair that determine whether the seeds shall be horny or waxy.

Both pairs have been used in numerous experiments in the past decade and the *C c* pair has always behaved in orthodox fashion. The *Wx wx* pair, on the other hand, though closely approximating a simple Mendelian behavior, has given, with some consistency, a deficiency of *wx wx* zygotes of 1 or 2 per cent.

Several explanations have been suggested to account for this slight deficiency but it now appears that these are inadequate, for not only are the *wx wx* zygotes often below the expected but under certain circumstances they are greatly in excess of the expected proportions.

From experiments with the storage of pollen it seems clear that in fresh pollen the effective gametes bearing the *wx* genes are below equality, whereas in old pollen such gametes are in excess of equality. The hitherto observed approximation of the percentage of waxy seeds to the Mendelian expected seems to have been the result of a fortuitous blending of fresh and old pollen, or to the even more remarkable coincidence—the use of pollen of just that age where the two sorts of gametes function equally well. In a certain sense the alteration in the proportion of the *Wx wx* genes in the stored gametes may be considered as selection, differentiating between gametes.

Although a legitimate difference of opinion exists as to the rôle of natural selection in evolution, there can be no question that stringent conditions result in the elimination of maladjusted organisms. Certain combinations of hereditary factors doom the individual possessing them to an early death, and still others make survival

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difficult except under the most favorable conditions. With plants the operation of natural selection is an ever-present and clearly visible phenomenon. This is well illustrated by those plants that inherit a genetic combination which prevents the development of chlorophyll. Geneticists are familiar with numerous heritable conditions that effect the vitality of the organism; some as in albinism, causing death at very early stages and others permitting the plants to struggle through to the production of a few seeds. Practically all mutations have a lower vitality than the normal forms from which they came, and are, therefore, more susceptible to adverse environment. Such cases illustrate natural selection operating upon zygotes.

From the standpoint of greatest economy of effort, a selection operating upon gametes offers many advantages. Especially is this true of plants, where millions of gametes are formed and exposed to external conditions for every one that functions successfully.

If external factors are to differentiate between gametes it is necessary that the characteristics of gametes be influenced by the genes they bear. The opportunity to measure directly any influence of the genes on the character of the gametes is limited. However, if it were found that adverse conditions operated as a selective agent in such a manner as to alter Mendelian proportions, it would be a fair inference that the genes had affected the characteristics of the gametes.

Not many examples, however, have been reported of selection operating upon gametes. It is true that selective fertilization is a well-established phenomenon and that certain classes of self and cross sterility involve gametic selection. These cases, however, all concern the interaction of the genetic factors of the gametes of opposite sexes and properly can not be considered as selective differentiation in the sense of external factors affecting unequally the different sorts of gametes.

Belling (1)<sup>2</sup> and others have shown that under normal conditions certain genetic factors are lethal to the gametes carrying them, but it is possible that under more favorable circumstances some of these gametes could effect fertilization. If this were possible such cases would provide an example of an external force operating as a selective agent upon gametes. However, no clearly authenticated examples of the revival of genetically sterile gametes, by unusually favorable conditions, have been reported and these cases must be left in the realm of possibility only.

In animals it has been shown that alcohol, when administered to prospective parents, acts as a selective agent not only on the newly constituted zygotes but on the gametes as well (12). The experiments with alcoholized animals furnish the best authenticated examples of selection operating upon the gametes. However, no evidence has been presented to show that specific genes ultimately determine the fate of the gamete bearing them. The evidence for selection rests upon such multiple gene characters as size and vigor as expressed by the offspring of alcoholized parents and the selective agent apparently operates upon a combination of genes.

With most genes their presence in the gamete can be inferred only from breeding tests, but in the case of the gene for waxy endosperm in the seeds of maize its presence in the gamete is associated with a

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 73.

chemical change that can be demonstrated by suitable treatment with iodine (9).

It follows that if the chemical nature of the contents of the gametes is influenced by the genes borne by them, an opportunity is presented for external factors to operate as selective agents. This would seem to be the case with the male gametes of maize, and evidence of the operation of selective factors is submitted in the following paper.

It has long been known that the percentage of waxy seeds is often below the expected in crosses where the male parents are heterozygous for this character. The conclusion was reached early that the male gametes bearing the waxy gene were at a disadvantage in effecting fertilization when in competition with gametes bearing the dominant allelomorph. Recently Brink has reported several experiments that confirm the conclusion as to the lower viability of male gametes bearing *wx* (3). His experiments show that the smaller proportion of functioning gametes bearing the waxy gene is due to a lower vitality of these gametes. This lower vitality is manifested by a slower rate of pollen-tube growth, when compared with that of the gametes bearing the dominant allelomorph.<sup>3</sup>

Working with comparable material but with a somewhat different viewpoint, the writer has amassed data that fail to support all of Brink's conclusions and that provide still further insight into the behavior of the gametes bearing the *wx* gene. These data have been brought together in the present report. Much of the material is of a disconnected nature, some being fragmentary, but in view of the continued interest in the waxy-nonwaxy segregation it was thought desirable to make all the data available.

Accordingly, the records accumulated over a period of several years have been scanned for their bearing upon the questions raised by others as well as upon those arising in these studies. That the results presented in the following pages occasionally fail to accord with those published by others simply indicates that knowledge of this pair of endosperm factors is as yet imperfect. Doubtless better agreement will follow further investigations, and it is to be hoped that the views here published will meet with sufficient incredulity to stimulate critical experimentation. The study of the waxy character has attained a respectable age, and without apparent loss of vigor, has retained a lively interest.

In the discussion of the behavior of the waxy gene it became distressingly obvious that the very awkward phrase "ratio of waxy to nonwaxy bearing gametes" recurred with disconcerting frequency. To avoid this undesirable expression, the term "nonwaxy" has been omitted wherever possible, and the phrase has been further shortened

<sup>3</sup> Since this paper was prepared, four articles have appeared bearing directly on the subject of gametic selection. Working with shape and color of the seeds in peas, Bond (2) shows that when immature stigmas are fertilized with mature pollen from the same plants the proportion of wrinkled to smooth seeds is in excess of the expected, whereas when flowers on the same plants are self-pollinated, wrinkled seeds are below the expected. This difference is attributed to the fact that pollen applied to immature stigmas is in effect stored, since germination is delayed until the stigmas are receptive.

Kiesselbach and Petersen (8) have added further data confirming the deficiency of waxy seeds. They conclude that the deficiency is due to a differential pollen tube growth, dependent upon the waxy gene or a gene closely linked with it.

Mangelsdorf and Jones (10), in a discussion of the gametophytic factors in plants, suggests that the deficiency of waxy seeds is due to the action of a gametophytic factor located on the chromosome with the waxy gene.

Muller and Settles (11) show that, at least for *Drosophila*, the functioning of the gametes is not affected by the genes they bear. These authors discuss the differences between plants and animals with respect to the sporophytic generation and point out that in the former, environic differences might be expected to result in gametic selection.

by characterizing the gametes by the nature of the genes borne by them, disregarding the fact that the gametes serve only as vehicles for the genes. It is believed that no serious ambiguity results from this abbreviated form of statement, and the reader is spared the repetition of an annoying phrase.

Before presenting the results obtained in deliberate attempts to modify the gametic ratios with respect to the *c* and *wx* genes an analysis will be presented of data, amassed for other purposes, which may be utilized to confirm or refute previous conclusions.

#### RATIO OF WAXY TO HORNY NOT INVARIABLY LOWER IN THE MALE GAMETES

In a previous paper (7) it has been shown that the proportion of waxy to horny seeds is less than the expected when the male parent is heterozygous for the waxy gene and, further, that when the female parent is heterozygous no such deficiency occurs. This conclusion was based on data, only a small part of which involved identical plant reciprocals, and was open to the criticism that the plants furnishing the gametes of one sex were not identical with those furnishing gametes of the other sex.

Recently, with more extensive material, Brink (3) has confirmed the conclusion with respect to the deficiency of waxy seeds when the male parent is heterozygous, but his data suffer from the defect mentioned above in that the comparison was not restricted to direct plant reciprocals.

In a study of the factors affecting the rate of crossing over there has been accumulated a series of 151 direct plant reciprocals from 13 closely related progenies. These reciprocal crosses provide material for a further test of the ratio of waxy to horny gametes in the sexes.

None of these ears involved, in a heterozygous condition, either the *su* endosperm or *I* aleurone factors that have been shown by Brink to affect the proportion of waxy gametes.

TABLE 1.—Comparison of the percentages of waxy gametes in the two sexes; crosses limited to plant reciprocals

Progeny designation	Number crosses	Female gametes		Male gametes		Difference, female—male
		Number of seeds	Per cent waxy	Number of seeds	Per cent waxy	
Dh 416 L3 L1 C1 L1 L22.....	4	1,919	49.4±1.21	1,885	51.1±1.00	-1.7±1.57
Dh 416 L3 L1 C1 L1 L1 L23.....	7	3,400	49.6±.41	2,104	49.8±.49	-.2±.64
Dh 416 L3 L1 C1 L1 L2 L23.....	8	3,064	51.2±.08	2,722	49.6±.77	1.6±1.03
Dh 416 L3 L1 C1 L1 L3 L23.....	7	2,933	48.5±.48	1,387	49.0±.72	-.5±.87
Dh 416 L3 L1 C3 L21.....	5	2,331	50.1±.46	4,100	56.0±1.55	-5.9±1.62
Dh 416 L3 L1 C3 L1 L22.....	5	3,296	50.0±.35	2,266	54.1±.63	-4.1±.72
Dh 416 L3 L1 C5 L21.....	5	4,068	48.7±.67	7,352	48.5±.53	.2±.85
Dh 416 L3 L1 C5 L4 L23.....	7	3,523	49.8±1.30	3,661	47.7±.77	2.1±1.51
Dh 416 L3 L1 C5 L4 L1 L24.....	17	7,336	49.2±.36	6,300	46.7±.59	2.5±.69
Dh 416 L3 L1 C5 L3 L1 R23.....	49	24,627	50.3±.23	27,517	48.1±.31	2.2±.39
Dh 416 L3 L1 C5 L3 L2 R23.....	11	5,284	49.4±.36	3,683	46.3±.79	3.1±.87
Dh 417 L1 L2 L1 L21.....	13	4,995	49.9±.56	4,693	50.2±.57	-.3±.80
Dh 417 L1 L2 L1 L2 L23.....	13	4,122	51.3±.70	3,757	49.3±.61	2.0±.93

\* All errors corrected for size of population.

The summarized data for the 13 progenies are presented in Table 1. Inspection shows that there is no consistent deficiency of functioning

waxy gametes in the male sex, and in fact the greatest differences between the sexes in this respect are those where a deficiency of waxy gametes is indicated in the female sex.

From inspection there would seem to be no objection to combining the results from the 13 progenies, but Fisher's (6) test for homogeneity clearly shows this array of differences to be heterogeneous.

It would appear then that the earlier conclusion as to the deficiency of waxy seeds when heterozygous plants are used as male parents is applicable only to certain progenies, and the opposite condition may obtain with other material.

However, despite the failure of this material to substantiate earlier conclusions, it is clear that the proportion of functioning waxy gametes in the male sex is more variable than the proportion in the female sex.

If the mean standard deviation of the percentage of waxy seeds is calculated for the two sexes in the reciprocal crosses from the 13 progenies it is found that the  $\sigma \text{ } \varnothing = 2.365 \pm 0.150$  and  $\sigma \text{ } \sigma = 2.98 \pm 0.118$ , or a difference of  $0.615 \pm 0.19$ . Thus the variability of the waxy-horny ratio in the male gametes is 26 per cent greater than that found in female gametes, but this difference only slightly exceeds three times the error.

If the data are treated as paired observations of the standard deviation of male and female gametes, and the mean difference and probable error calculated from the array of differences between the members of each pair, weighted by the reciprocal of the squared probable error of the differences (to correct for populations of varying sizes), the mean difference in standard deviation is found to be  $0.572 \pm 0.173$ , a difference of 3.3 times the probable error (when the standard deviation of the array has been corrected for the small size of the population). This latter method of treating the paired observations corresponds very closely to Student's method. The latter shows that a difference of the observed magnitude would not be expected to occur as the result of chance more often than once in 42.5 trials. This is very close to the odds of 37.5 to 1 deduced from the departure of 3.3 times the error.

Further, Fisher's test (6) for homogeneity shows that the array of differences is homogeneous.

If there is any intimate connection between the proportion of functioning waxy gametes and the variability of the percentage of such gametes, it should be found that the percentage of waxy gametes is more variable in the male sex when there is a deficiency of such gametes in this sex and, conversely, the variability should be less in the males when the waxy gametes are in excess in this sex. No such agreement is found, and in fact the most variable progeny with respect to the waxy ratio in the male gametes is one in which this ratio was highest in the male gametes.

It has been shown in a previous publication (5) that the ratio of waxy to nonwaxy gametes in the male sex is profoundly modified by storage of the pollen for even a few hours, and it seems not unreasonable to infer that shorter periods of storage would have a correspondingly less pronounced effect.

In these experiments the tassels of the plants to be used as male parents were bagged usually one or more days before making the crosses. There is evidence that a small quantity of pollen may remain viable

for 24 hours, and since old pollen has a higher proportion of waxy gametes, there would be a tendency to increase the percentage of waxy seeds over the expected equality, when the parents had been bagged even one day in advance of use. An effect of this nature might well account for the observed gametic ratios in the sexes. It appears that the percentage of waxy gametes is more variable in the male than in the female, but there is no evidence that this variability is associated with a differential viability.

#### PERCENTAGE OF WAXY SEEDS ALIKE ON UPPER AND LOWER EARS WHEN MALE PARENT IS HETEROZYGOUS

Since in some instances the percentage of waxy seeds is higher when the male parent is heterozygous and in others when the female parent is heterozygous, it seemed desirable to examine other groupings of data not based on sex differences.

TABLE 2.—Percentage of waxy in upper and lower ears where male parent is heterozygous (Dh 416 L3 L1 C5 L4 L1 L24)

Plant numbers	Upper ears		Lower ears		Difference Upper— lower
	Total seeds	Per cent waxy	Total seeds	Per cent waxy	
5702×5905.....	379	46.4±1.73	82	52.4±3.72	-6.0±4.11
5710×5909.....	170	44.7±2.57	340	50.0±1.83	-5.3±3.16
5725×5931.....	660	49.9±1.31	593	49.8±1.39	.1±1.91
5759×5912.....	598	45.7±1.37	583	47.4±1.40	-1.7±1.96
5718×5903.....	516	50.8±1.48	566	48.1±1.42	2.7±2.05
5743×5950-n.....	564	54.1±1.42	703	50.1±1.27	4.0±1.91
5781×5972.....	525	54.7±1.47	568	55.1±1.41	-.4±2.08
5795×5991.....	266	53.4±2.06	489	54.0±1.52	-.6±2.56
3601×3997.....	518	46.7±1.48	181	55.2±2.50	-8.5±2.91
3620×3998.....	364	43.1±1.75	628	43.0±1.33	.1±2.19
3631×3998.....	596	48.8±1.38	592	49.0±1.39	-.2±1.96
3664×4022.....	491	42.2±1.50	344	51.4±1.82	-9.2±2.36
Mean.....					-1.03±.78

TABLE 3.—Percentage of waxy in upper and lower ears where male parent is heterozygous (Ph 230 L1 R24)

Plant numbers	Upper ears		Lower ears		Difference Upper— lower
	Total seeds	Per cent waxy	Total seeds	Per cent waxy	
510×616.....	547	41.7±1.42	282	42.9±1.99	-1.2±2.45
519×654.....	258	41.5±2.07	226	49.6±1.57	-8.1±2.61
533×723.....	46	52.2±4.97	402	47.0±1.68	5.2±5.25
540×720.....	298	50.0±1.96	324	44.1±1.86	5.9±2.71
546×707.....	423	42.8±1.63	260	41.9±2.07	.9±2.63
548×603.....	394	40.6±1.67	407	40.3±1.64	.3±2.34
1×101.....	486	41.8±1.51	509	40.1±1.46	1.7±2.11
3×110.....	462	51.4±1.06	319	46.3±1.41	5.1±1.76
5×200.....	128	46.1±2.98	317	51.4±1.90	-5.3±3.53
6×103.....	708	47.7±1.27	190	36.3±2.36	11.4±2.68
7×136.....	429	49.0±1.62	528	41.8±1.45	7.2±2.18
12×162.....	274	46.0±2.06	229	45.0±2.22	1.0±3.01
13×149.....	8	37.5±10.55	22	36.4±6.92	1.1±13.48
15×126.....	193	45.1±2.42	36	47.2±5.62	-2.1±6.08
23×169.....	265	49.4±2.07	274	46.4±2.03	3.0±2.90
27×181.....	534	49.4±1.46	415	42.2±1.63	7.2±2.19
28×182.....	465	46.2±1.56	511	52.6±1.49	-6.4±2.16
Mean.....					2.03±.85

The records afford two progenies in which upper and lower ears were pollinated at the same time with identical lots of heterozygous pollen. The ears are shown in Tables 2 and 3. In neither case do the ears of one position have a percentage of waxy significantly different from those in another position, and in one progeny the difference is in the direction of the upper ears having the highest percentage, while in the other the lower ears have the highest percentage.

PERCENTAGE OF WAXY SEEDS HIGHER ON UPPER THAN ON LOWER EARS WHERE FEMALE PARENT IS HETEROZYGOUS

A third progeny provides data that may be used to detect a difference between upper and lower ears when the female parent was heterozygous and the male parent was homozygous for *wx*.

The ears of this nature are shown in Table 4. The ears were pollinated at the same time, each pair with identical pollen, and the only difference between the members of a pair is that due to their position. The difference in percentage waxy is  $2.11 \pm .45$ , with the upper ears having the highest percentage. This difference is fully as large as any found in groupings based on sex.

TABLE 4.—Percentage of waxy in upper and lower ears where female parent is heterozygous (progeny Dh 416 L3 L1 C5 L4 L1—4—L24)

Plant number	Upper ears			Lower ears			Difference
Heterozygous parent	Total seeds	Number waxy	Per cent waxy	Total seeds	Number waxy	Per cent waxy	Upper-lower
3984.....	560	275	49.1 $\pm$ 1.42	154	71	46.1 $\pm$ 2.70	3.0 $\pm$ 3.05
3985.....	698	360	51.6 $\pm$ 1.27	448	210	46.9 $\pm$ 1.59	4.7 $\pm$ 2.04
3986.....	813	403	49.6 $\pm$ 1.18	441	214	48.2 $\pm$ 1.60	1.4 $\pm$ 1.99
3988.....	495	238	48.1 $\pm$ 1.51	61	30	49.2 $\pm$ 4.31	-1.1 $\pm$ 5.35
3992.....	166	91	54.8 $\pm$ 2.61	86	50	58.1 $\pm$ 3.57	-3.3 $\pm$ 4.42
3993.....	245	119	48.6 $\pm$ 2.15	276	120	43.5 $\pm$ 2.01	5.1 $\pm$ 2.94
3994.....	355	190	53.5 $\pm$ 1.79	444	235	52.9 $\pm$ 1.60	.6 $\pm$ 2.40
3996.....	434	217	50.0 $\pm$ 1.62	27	14	51.8 $\pm$ 6.50	-1.8 $\pm$ 6.70
3998.....	349	176	50.4 $\pm$ 1.75	423	215	50.8 $\pm$ 1.54	-.4 $\pm$ 2.33
3999.....	242	127	52.5 $\pm$ 2.16	554	284	51.3 $\pm$ 1.43	1.2 $\pm$ 2.50
4000.....	188	86	45.7 $\pm$ 2.45	15	7	46.7 $\pm$ 8.70	-1.0 $\pm$ 9.02
4004.....	118	55	46.6 $\pm$ 2.89	249	131	52.6 $\pm$ 2.13	-6.0 $\pm$ 3.59
4008.....	555	276	49.7 $\pm$ 1.43	454	218	48.0 $\pm$ 1.58	1.7 $\pm$ 2.13
4009.....	397	199	50.1 $\pm$ 1.69	355	164	46.2 $\pm$ 1.78	3.9 $\pm$ 2.46
4010.....	136	67	49.3 $\pm$ 2.89	410	198	48.3 $\pm$ 1.66	1.0 $\pm$ 3.33
4020.....	410	211	47.8 $\pm$ 1.66	119	59	52.1 $\pm$ 3.08	-4.3 $\pm$ 3.50
4022.....	502	260	51.6 $\pm$ 1.50	306	150	44.1 $\pm$ 1.91	7.5 $\pm$ 2.43
Mean.....			50.0 $\pm$ .31			48.9 $\pm$ .52	2.11 $\pm$ .45

Upper ears are usually somewhat more advanced than the lower ears, and when both are pollinated at the same time the gametes of the upper ears are at least some hours older than those of the lower ears. If the female gametes are affected by age in the same manner as are the male gametes then the upper ears would be expected to have a higher percentage of waxy than the lower ears.

Although the upper ears in this material have a higher percentage of waxy seeds, this does not conflict with Brink's (3) evidence that long styles tend to impair the functioning of waxy-bearing gametes. It is true that ears with long styles are necessarily older than those with short styles, and for this reason would be expected to have a higher percentage of waxy if the results shown in Table 4 are of

general application. However, in Brink's experiments the short-styled controls were obtained by cutting back silks which presumably were of the same length as those used for the long-style parents, and hence the gametes were of the same age. However, it should be noted that in both the long and short styled material the percentage of waxy was below the expected, whereas both sets of ears must have been older at the time of fertilization than normal material, and hence would be expected to have an excess of waxy seeds. A possible explanation of the failure to obtain a higher percentage of waxy on these ears lies in the fact that they were self-pollinated. If the ratio of waxy to nonwaxy in the effective male gametes were less than equality, a condition often met with, this inequality may have more than overbalanced any inequality in the opposite direction resident in the female gametes.

There is no occasion, however, to overstrain the comparison of Brink's experiment with that presented in Table 4, since many more data of a substantiating nature would be necessary to establish the generality of the phenomenon of an increase in effective waxy female gametes with advancing age.

#### PERCENTAGE OF WAXY SEEDS ALIKE ON BUTTS AND TIPS OF THE EAR

If the pollen tubes of gametes bearing the waxy gene are at a disadvantage in effecting fertilization as compared with those bearing the nonwaxy gene, then it should be found: (1) That the percentage of waxy seeds on the lower halves of the ears is less than that on the upper halves when heterozygous pollen is applied to double recessive silks, but no such difference should be found when double recessive pollen is applied to the silks of heterozygous plants; (2) a correlation should be found between the total number of seeds on an ear and the percentage of waxy seeds when heterozygous pollen is used on double recessive plants, but no correlation is expected where the procedure is reversed, i. e., with homozygous waxy-bearing pollen applied to heterozygous plants.

TABLE 5.—Percentage of waxy seeds in tip and base of ears where male parent is heterozygous (progeny Dh 416 L3 L1 C5 L3 L1 R23)

Plant number	Tip		Base		Difference Tip—base
	Total seeds	Per cent waxy	Total seeds	Per cent waxy	
6005.....	205	53.7±2.35	106	56.6±3.37	-2.9±4.11
6012.....	140	42.9±2.85	201	44.3±2.37	-1.4±3.71
6014.....	202	50.0±2.37	180	44.4±2.51	5.6±3.45
6017.....	160	50.0±2.67	140	50.7±2.85	-7.3±3.91
6024.....	272	39.7±2.05	220	38.6±2.28	1.1±3.07
6016.....	170	47.1±2.59	123	42.3±3.05	4.8±4.00
6010.....	225	38.2±2.23	213	43.7±2.31	-5.5±3.21
6025.....	120	37.5±3.08	149	45.6±2.76	-8.1±4.14
Mean.....					-0.72±.29

Eight ears are available to test the hypothesis in (1), where heterozygous pollen was applied to double recessive plants (Table 5).

These 8 ears comprise 2,826 seeds. They were divided into upper and lower halves and the two lots classified separately. The ears were not long, but the pollen tubes functioning on the lower halves must have grown on an average about 3 inches further than those fertilizing the ovules of the upper halves of the ears.

The differences between the eight pairs of tips and butts were treated as an array from which the mean and standard deviation (corrected) were calculated; both being weighted by  $1/E_d^2$  to correct for differences in number of seeds. Treated in this manner, the mean difference in percentage of waxy seeds between tips and butts is found to be  $0.724 \pm 0.29$  per cent; the butts having on an average 0.7 per cent more waxy seeds than the tips. The difference is not significant, and with the error involved a difference smaller than 1 per cent could not have been established. Since the observed percentage of waxy seeds in Brink's (3) long-styled material was 20.44, it follows that 40.8 per cent of the functioning male gametes bore the *wx* gene, whereas in the control short-style material the observed percentage of waxy seeds was 23.95, showing that 47.9 per cent of the functioning male gametes bore the *wx* gene. The difference may be taken to represent the differential effect of the long styles; being in this case 7.1 per cent, or approximately 2 per cent for each increase of 3 inches in length of style above the control series.

Forty-two ears are available to test the second hypothesis of (1) above, where homozygous recessive *wx* pollen is applied to heterozygous plants. These ears fell into four classes with respect to color, and the data have been summarized in Table 6. The mean difference for each color class was determined separately by the method outlined above. Treating the array of differences of the paired values in the several color classes in the same manner as above, the mean difference is found to be  $0.132 \pm 0.55$ , with the upper halves of the ear having the highest percentage of waxy seeds. There is little indication, therefore, that the percentage of waxy seeds is higher in the tips than in the butts of ears.

TABLE 6.—Difference in percentage of waxy seeds in tip and base of ears where female parent is heterozygous (progeny Dh 416 L3 L1 C5 L3 L1-4-R23)

Nature of cross	Number of ears	Tip-base, mean difference in per cent waxy
Back crossed, 50.0 per cent white.....	23	$-1.33 \pm 0.73$
Back crossed, 62.5 per cent white.....	9	$1.81 \pm 1.32$
Self, 25.0 per cent white.....	3	$1.44 \pm 1.62$
Self, 43.75 per cent white.....	7	$2.92 \pm .84$
Mean.....		$.132 \pm .55$

The correlation testing (3) above has been published (5). Considering male gametes alone, a coefficient of  $0.161 \pm 0.47$  is found between the percentages of waxy and the total number of seeds formed when heterozygous waxy pollen is applied, whereas a coefficient of  $-0.017 \pm 0.73$  is found between these two variables when the female parent is heterozygous for waxy.

Restricting the population to identical plant reciprocals, and thus insuring the consideration of the same plants in measuring the relationship in the two sexes, the following coefficients are found:

	Per cent waxy in male	Per cent waxy in female
Number of seeds on male.....	0.017 $\pm$ 0.98	0.090 $\pm$ 0.97
Number of seeds on female.....	.114 $\pm$ .86	.020 $\pm$ .98

None of these coefficients is significant. It is of interest to observe that in this series of 47 reciprocal crosses between sibs, the correlation between the percentage of waxy seeds when the heterozygous parent is used as a female and that found when these same plants serve as male parents is  $-0.020 \pm 0.98$ , thus showing that the variations in the percentage of waxy are not due to genetic diversity.

#### LONG STYLES NOT ASSOCIATED WITH A LOW PERCENTAGE OF WAXY SEEDS

Brink (3) has shown that in two lots of self-pollinated ears differing in style length the percentage of waxy seeds is lower in the group having the longest styles.

In the course of an investigation into the factors affecting crossover percentages, paired back-cross pollinations were made on ears of long and short styles and on ears having styles of the same length. In these cases a single plant in each instance furnished the pollen for a given pair of ears.

The data accumulated for the crossover study will serve also to determine the general applicability of Brink's conclusion.

TABLE 7.—Comparison of the percentage of waxy seeds on ears differing in length of styles and on ears where the styles were of the same length; these ears were borne on homozygous recessive plants

EARS DIFFERING IN SILK LENGTH					
Long-styled ears		Short-styled ears		Difference	
Length of silk (inches)	Per cent waxy	Length of silk (inches)	Per cent waxy	Length of silk (inches)	Per cent waxy; long-short
3	51.4 $\pm$ 2.81	$\frac{3}{4}$	46.1 $\pm$ 4.41	$2\frac{1}{4}$	5.3 $\pm$ 5.22
4	49.4 $\pm$ 3.07	2	46.4 $\pm$ 3.01	2	3.0 $\pm$ 4.30
3	41.8 $\pm$ 2.30	2	51.4 $\pm$ 1.88	1	-9.6 $\pm$ 2.97
3	46.2 $\pm$ 2.31	1	52.6 $\pm$ 2.21	2	-6.4 $\pm$ 3.19
3	41.5 $\pm$ 3.07	1	49.6 $\pm$ 3.33	2	-8.1 $\pm$ 4.53
7	42.8 $\pm$ 2.41	4	41.9 $\pm$ 3.06	3	.9 $\pm$ 3.90
7	40.6 $\pm$ 2.47	2	40.3 $\pm$ 2.43	5	.3 $\pm$ 3.46
3	46.0 $\pm$ 3.01	2	45.0 $\pm$ 3.29	1	1.0 $\pm$ 4.46
Mean				2.16	-2.95 $\pm$ 1.36
EARS OF SAME SILK LENGTH					
1	49.0 $\pm$ 2.41	1	41.8 $\pm$ 2.15	0	7.2 $\pm$ 3.23
1	47.7 $\pm$ 1.88	1	36.3 $\pm$ 3.49	0	11.4 $\pm$ 3.97
1	41.7 $\pm$ 2.11	1	42.9 $\pm$ 2.95	0	-1.2 $\pm$ 3.63
1	50.0 $\pm$ 2.90	1	44.1 $\pm$ 2.76	0	5.9 $\pm$ 4.01

Considering the cases where the male parent was heterozygous for *wx* and the female was homozygous recessive for this factor, the results shown in Table 7 were found. It is apparent that the percentage of waxy seeds on the long-styled ears was lower than that on the short-styled ears, but the same sort of differences are observed where the styles of the pair were of equal length. When large differences within the pair are found they do not seem to be correlated with large differences in silk length.

The difference between the percentage of waxy in the long and short-styled series is not significant, but in amount accords well with that observed by Brink. The failure to find a better agreement between the difference in length of style and that in percentage of waxy raises the question as to whether in this comparison length of style had any effect on the functioning of the gametes having the *wx* gene.

A larger series involving style length where the female parent was heterozygous and the male homozygous *wx* are shown in Table 8.

TABLE 8.—Comparison of the percentage of waxy seeds on ears differing in length of styles and on ears where the styles were of the same length; these ears were borne on heterozygous plants

EARS DIFFERING IN SILK LENGTH					
Long-styled ears		Short-styled ears		Difference	
Length of silk (inches)	Length of waxy	Length of silk (inches)	Per cent waxy	Length of silk (inches)	Per cent waxy, long-short
5	46.4±1.91	4	47.4±4.30	1	-1.0±4.70
1	45.4±2.85	1½	48.1±2.59	1½	-2.7±3.85
3	45.7±2.50	1	50.7±6.11	2	½5.0±6.60
4	45.2±2.74	¾	50.2±2.51	¾	-5.0±3.72
8	49.5±2.05	¾	47.6±2.13	7¾	1.9±2.95
3	51.0±2.34	1	53.4±4.10	2	-2.4±4.72
8	49.5±1.93	4	48.6±2.73	4	.9±3.34
3	50.3±3.94	1	53.08±2.95	2	½3.5±4.92
3	52.2±2.02	1	47.1±2.60	2	5.1±3.29
2	50.0±3.20	1	53.6±2.54	1	-3.6±4.08
4	52.2±2.22	1½	46.4±3.06	2½	5.8±3.77
4	53.4±2.79	1	51.7±4.60	3	1.7±5.38
5	47.6±2.60	3	47.5±4.52	2	.1±5.21
5	50.5±2.28	4	45.5±2.65	1	5.0±3.50
4	50.7±2.60	1½	48.5±5.02	3½	2.2±5.64
4	45.6±2.18	1	45.7±2.55	3	-.1±3.35
6	51.5±2.16	4	48.4±2.31	2	3.1±3.16
5	49.3±2.55	1	49.4±2.33	4	-.1±3.43
3	52.0±2.79	1	50.2±3.35	2	1.8±4.35
1	48.9±2.61	½	48.5±2.71	½	.4±3.76
Mean				2.30	.76±.49
EARS OF SAME SILK LENGTH					
2	51.4±2.57	2	54.7±4.40	0	-3.3±5.00
2	53.3±3.88	2	56.4±3.01	0	-3.1±5.01
1½	50.7±6.02	1½	50.5±5.52	0	-.8±8.16
1	48.6±2.35	1	52.8±4.46	0	-4.2±5.04
2	36.7±6.22	2	44.4±3.70	0	-7.7±7.22
1	49.7±2.70	1	51.8±2.64	0	-2.1±3.78
1	49.2±2.36	1	48.4±2.46	0	.8±3.41
1	46.7±2.78	1	44.4±4.46	0	2.3±3.26
2	49.4±4.00	2	49.3±1.82	0	.1±4.30
1	51.6±2.58	1	55.3±5.70	0	-3.7±3.15
3	50.8±3.27	3	51.3±5.73	0	-.5±3.73

Here again no significant difference is found either when the pairs differ in style length or when they have styles of the same length.

Variations in the percentage of waxy seeds are found, but these do not seem to be associated with differences in style length.

From the standpoint of differential pollen-tube growth no effect of style length is to be expected in the series of ears shown in Table 8, but it will be observed that differences in the percentage of waxy are found that are entirely comparable with those observed in the series shown in Table 7, where heterozygous pollen was used.

In none of these cases, however, were the differences in style length as great as those of Brink, and the failure to corroborate his conclusions may well be due to this fact. Nevertheless, it should be observed that there are many ears where the waxy class is deficient in numbers, though no great length of style is involved.

#### GAMETES BEARING THE GENES *SU* AND *WX* NO LESS EFFECTIVE THAN THOSE WITH OTHER COMBINATIONS OF THESE GENES

Brink (3) found that when plants homozygous for *su* and heterozygous for *Wx* were self-pollinated only 18.84 per cent of the resulting seeds were waxy, whereas the expected percentage was 25. Recognizing that some factor other than *su* may have been involved in his results, he states, "there is considerable justification for the conclusion that the gene for sugary greatly increases the disparity between the observed proportion of nonwaxy and waxy seeds and that expected on a chance basis."

To obtain a deficiency of 6.26 per cent on self-pollinated ears, if the assumption be made that both sexes are affected equally, the gametic proportions must be 56.6 nonwaxy to 43.4 waxy. But if it be assumed that the distorted ratios result from some sort of differential viability operative only in the male gametes, then 25 per cent of the waxy-bearing gametes fail to function, and the effective ratio of nonwaxy to waxy gametes is 62.5 to 37.5.

Since Brink has shown that gametes bearing *wx* are less effective than those bearing *Wx* when the styles are long, it is entirely possible that the pollen tubes of *su wx* gametes have a slower rate of growth than those constituted *Su wx*. If this were a general phenomenon the ratios obtained in all crosses involving *Su* and *Wx* should be unorthodox.

As a first check on the hypothesis of a differential viability of the genes bearing *su wx*, the published data may be examined. Collins and Kempton (4) published the seed classes of 51 ears that were the result of self-pollinating plants heterozygous for *Su* and *Wx*. At the time these data were published no method was known for determining the *su wx* group other than breeding tests, and these accordingly were combined with the *su Wx* group, making the expected on a normal hypothesis 9 horny, 3 waxy, 4 sweet. If it be assumed that one-fourth of the *su wx* gametes fail to function, as in Brink's case, the expectation for these ears becomes 9.42 horny, 2.915 waxy, 3.665 sweet.

The observed distribution was:

	Horny	Waxy	Sweet
Observed.....	12, 728	3, 941	5, 463
Expected 9-3-4.....	12, 449	4, 150	5, 533
	+279	-209	-70

$$\chi^2 = 17.66$$

The departure from the 9-3-4 ratio is too large to be attributed to chance, but since there is a priori knowledge from many other progenies of a deficiency of waxy seeds and, further, since the observed departures are in the classes horny and waxy, the failure to obtain a better fit is clearly due to the defect of the waxy.

Knowing that waxy seeds are generally deficient, and having an observed deficiency in this class of 209 seeds, it is apparent that if the same percentage of defect held for the waxy gene when in combination with *su* the expected deficiency in the *su wx* class would be 69.75 seeds. In the case above the *su wx* class was combined with the *su Wx* class, and this combination was in defect 70 seeds. It is clear that if this whole amount is assumed to be due to a defect of the *su wx* class it is no larger than would have been expected from the behavior of the *Su wx* group, and therefore offers no evidence for a lower viability of gametes the *su wx* combination of genes as compared with those bearing *Su wx*.

Comparing the observed population with that expected on the assumption of a 25 per cent mortality of the *su wx* gametes the following is obtained:

	Horny	Waxy	Sweet
Observed.....	12, 728	3, 941	5, 463
Expected.....	13, 026	4, 034	5, 072
	-298	-93	+391

$$\chi^2 = 46.19$$

Clearly this fit is much worse than that obtained in the 9-3-4 comparison.

More recent material may be used to examine this matter further. These data are confined to two sister  $F_2$  progenies (Ph 230 L1 and Ph 230 L2).

As the result of crosses of the nature *Su su wx wx* ♀ × *Su su Wx wx* ♂, 29 ears having 7,974 seeds were obtained. These were distributed:

	Horny	Waxy	Sweet	Waxy sweet
Observed.....	3, 220. 00	2, 632. 00	1, 170. 00	952. 00
Expected 3-3-1-1.....	2, 990. 25	2, 990. 25	996. 75	996. 75
	+229. 75	-358. 25	+173. 25	-44. 75

$$\chi^2 = 84.86$$

A very poor fit, indeed, but one again due largely to the defect of the waxy seeds. Any deficiency in effective *su wx* gametes should be indicated by a lower percentage of waxy seeds in the sweet group when compared with that in the nonsweet group. The percentage is 44.86 in the former and 44.98 in the latter.

The disturbance in these ratios seems to be confined largely to the male gametes, since where the cross is made *Su su Wx wx* ♀ × *Su su wx wx* ♂ the fit to a 3-3-1-1 ratio is as good as could be expected, as is seen in the following comparison:

	Horny	Waxy	Sweet	Waxy sweet
Observed-----	5, 881. 00	5, 753. 00	2, 023. 00	1, 944. 00
Expected-----	5, 850. 37	5, 850. 37	1, 950. 10	1, 950. 10
	+30. 60	-97. 37	+72. 90	-6. 1

$$X^2 = 4.5$$

$$P = 0.25$$

Since in these cases abnormal waxy or waxy sweet behavior is being sought, it may be well to consider these two seed classes irrespective of the sweet class. On the hypothesis that the gametes bearing *su wx* are less able to effect fertilization than those with other combinations of these genes, the percentage of both waxy and waxy sweet seeds should be reduced. Thus in back crosses of the nature *Su su wx wx* ♀ × *Su su Wx wx* ♂ the double recessive class, *su wx* should be less than 12.5 per cent.

The progenies considered above provide 90 ears, in which 12.5 per cent of the seeds are expected to be of the double recessive class, *su wx*. These ears were obtained in the two sister progenies Ph 230 L1 and L2 by crossing plants of the nature *Su su wx wx* and *Su su Wx wx*. The percentage of waxy sweet seeds obtained with heterozygous pollen in progeny Ph 230 L1 is  $11.95 \pm 0.44$  and when heterozygous plants of this progeny are used as female parents the percentage is  $12.45 \pm 0.19$ . The heterozygous male gametes of progeny Ph 230 L2 gave  $11.93 \pm 0.46$  per cent of *su wx* seeds, whereas heterozygous female gametes of this progeny gave  $12.77 \pm 0.35$  per cent *su wx*. None of these percentages depart significantly from the 12.5 per cent expected if the *su wx* gametes functioned normally, but there is an indication that the percentage of *su wx* seeds is lower when the male parent is heterozygous than when the female is heterozygous.

To test this point further resort must be had to identical plant reciprocals, and these accordingly have been selected from the 90 ears and grouped in Table 9. Only 10 pairs of reciprocals are available, and these show a mean difference of  $1.69 \pm 0.32$ , with the percentage of waxy sweet seeds highest where the female parent is heterozygous.

If the assumption be made that 25 per cent of the gametes bearing the combination *su wx* fail to function, then back-crosses of the nature *Su su wx wx* ♀ × *Su su Wx wx* ♂ should give 45.8 per cent of the seeds waxy. As a test of this hypothesis Tables 10 and 12 have been prepared, the data being assembled from progenies Ph 230 L1 and L2.

Presumably the effect of the *su* gene on the survival of the gametes bearing the waxy genes can come into operation only in the male gametes. Table 10 presents 19 ears where the male gametes are measured. The mean percentage of waxy seeds on these ears is  $45.0 \pm 0.60$ , not a bad fit to the 45.8 per cent expected on the hypothesis that 25 per cent of the waxy sweet gametes fail to function.

TABLE 9.—Percentage of seeds of the double recessive combination waxy and sweet in plant reciprocals

Plant numbers	Male gametes		Female gametes		Difference
	Total seed	Per cent <i>su wx</i>	Total seeds	Per cent <i>su wx</i>	Female—male
1×101.....	995	10.5±0.66	727	13.5±0.86	3.0±1.08
8×162.....	728	10.2±.76	708	12.7±.85	2.5±1.14
13×149.....	22	18.2±5.54	797	13.7±.82	-4.5±5.60
20×192.....	270	12.6±1.36	711	11.8±.82	-.8±1.59
23×169.....	539	11.5±.93	1,144	12.2±.65	.7±1.14
506×624.....	68	8.8±2.32	148	11.6±1.77	2.8±9.23
513×746.....	394	10.4±1.04	49	12.2±3.16	1.8±3.32
519×654.....	484	10.5±.94	401	11.5±1.07	1.0±1.42
522×644.....	187	11.8±1.59	310	14.8±1.36	3.0±2.09
535×744.....	86	11.6±2.33	194	14.4±1.70	2.8±2.88
Mean.....					1.69±.32

TABLE 10.—Percentage of waxy seeds on ears segregating for *Su* (male gametes of Ph 230 L1 and L2)

Plant numbers	Total seeds	Number waxy	Per cent waxy	Plant number	Total seeds	Number waxy	Per cent waxy
1×101.....	995	407	40.9±1.05	513×746.....	394	181	45.9±1.69
5×200.....	445	219	49.2±1.60	514×646.....	574	241	42.0±1.39
8×162.....	728	324	44.5±1.24	516×611.....	350	152	43.4±1.78
12×162.....	503	229	45.5±1.49	519×654.....	484	219	45.2±1.53
20×192.....	270	124	45.9±2.04	520×671.....	187	88	47.0±2.46
23×169.....	539	258	47.9±1.46	532×693.....	124	68	54.8±3.02
28×182.....	976	484	49.6±1.08	535×744.....	86	33	38.4±3.54
501×624.....	118	56	47.4±3.10	535×743.....	270	122	45.2±2.04
503×637.....	348	122	35.0±1.72				
506×624.....	68	35	51.5±4.08				
507×657.....	65	28	43.1±4.14	Mean.....			45.0±.59

However, as has been seen, there are often significant differences in the sexes in the proportion of waxy gametes, and with the ears shown in Table 10 we may be dealing with some such phenomenon. As a check the ears shown in Table 11 have been assembled. These ears do not involve the *Su* gene in a heterozygous condition, and resulted from crosses of the nature *Su Su wx wx* ♀ × *Su Su Wx wx* ♂. The plants bearing them were sibs of those producing the ears in Table 10. The 19 ears in Table 11 have a mean of  $44.6 \pm 0.60$  per cent waxy, a percentage very close, indeed, to that found for the ears in Table 10, thus disposing of the hypothesis that the low percentage found for the ears in Table 10 was due to the lower survival rate of *su wx* gametes in plants heterozygous for *Su* and *Wx*.

The percentage of waxy seeds obtained on ears where the female parent was heterozygous for *Su* and *Wx*, and the male parent was heterozygous for *Su* but homozygous for *wx*, should not depart from the expected equality; and the ears shown in Table 12 are the result

of crosses of this nature. The percentage of waxy is  $49.6 \pm 0.19$ , a very close approximation to the expected 50 per cent.

TABLE 11.—Percentage of waxy seeds on ears not segregating for *Su* (male gametes *Ph 230 L1-1-R24* and *L2*)

Plant numbers	Total seeds	Number waxy	Per cent waxy	Plant numbers	Total seeds	Number waxy	Per cent waxy
3×110.....	781	358	45.8±1.20	510×616.....	829	349	42.1±1.16
4×232.....	570	264	46.3±1.41	517×611.....	243	137	56.4±2.15
6×103.....	898	407	45.3±1.12	527×739.....	295	154	52.2±1.96
7×136.....	957	431	45.0±1.09	531×721.....	154	78	50.6±2.71
15×126.....	229	104	45.4±2.22	531×677.....	274	128	46.7±2.04
16×157.....	450	167	37.1±1.55	540×720.....	692	293	42.3±1.26
17×157.....	554	272	49.1±1.43	546×707.....	683	290	42.4±1.27
25×147.....	172	83	48.2±2.56	548×603.....	801	324	40.4±1.17
27×181.....	949	439	46.2±1.09				
33×184.....	329	120	36.5±1.79	Mean.....			44.6±.60
504×624.....	42	17	40.5±5.10				

TABLE 12.—Percentage of waxy seeds on ears segregating for *Su* (female gametes *Ph 230 L1* and *L2*)

Plant numbers	Total seeds	Number waxy	Per cent waxy	Plant numbers	Total seeds	Number waxy	Per cent waxy
101×1.....	727	365	50.2±1.25	251×20.....	198	107	54.0±2.39
103×1.....	1,099	544	49.5±1.02	252×806.....	499	249	49.9±1.51
108×807.....	649	323	49.8±1.32	624×506.....	148	82	55.4±2.76
117×1.....	1,016	482	47.4±1.06	644×522.....	310	158	51.0±1.91
144×29.....	884	412	46.6±1.13	645×514.....	666	335	50.3±1.31
149×13.....	797	381	47.8±1.19	650×533.....	256	134	52.3±2.10
158×7.....	720	345	47.9±1.25	654×519.....	401	203	50.6±1.68
160×33.....	908	448	49.3±1.12	671×513.....	833	403	48.4±1.17
162×8.....	708	345	48.7±1.27	674×807.....	374	194	51.9±1.75
163×28.....	1,005	503	50.0±1.06	676×527.....	266	138	51.9±2.06
164×13.....	858	419	48.8±1.15	677×519.....	576	285	49.5±1.40
169×23.....	1,144	556	48.6±.99	680×527.....	102	43	42.2±3.30
187×33.....	436	241	55.3±1.61	683×527.....	284	156	54.9±2.00
192×20.....	711	362	50.9±1.27	697×523.....	102	51	50.0±3.34
200×808.....	636	326	51.2±1.33	713×535.....	238	112	47.0±2.18
203×14.....	281	149	53.0±2.02	744×535.....	194	92	47.4±2.42
216×5.....	1,004	494	49.2±1.06	746×513.....	49	24	49.0±4.81
222×1.....	978	492	50.3±1.08	748×535.....	137	68	49.6±2.88
224×1.....	683	338	49.5±1.29	749×535.....	369	184	49.9±1.75
234×36.....	376	189	50.3±1.74				
234×36.....	180	90	50.0±2.50	Mean.....			49.6±.19
239×33.....	533	258	48.4±1.46				

In Table 13 are listed the ears produced by crosses of the nature *Su Su Wx wx* ♀ × *Su Su wx wx* ♂. The plants producing these ears are sibs of those that bore the ears shown in Tables 10, 11, and 12. No departure from equality in the proportion of waxy to nonwaxy seeds is expected and none was found, the percentage being  $48.9 \pm 0.37$ .

It is apparent from these tables that in this progeny the percentage of waxy seeds resulting from the use of heterozygous pollen on plants homozygous recessive for this character is below equality, and less than that obtained where the crosses are reversed, i. e., the male parent homozygous recessive and the female heterozygous for waxy.

To determine this point directly recourse may be had to direct plant reciprocals. These are shown in Tables 14 and 15. Table 14 lists reciprocal crosses where both *Su* and *Wx* are involved, and Table 15 lists only those crosses where *Su* was homozygous dominant. In both instances the proportion of effective waxy gametes is highest

in the female. It should be observed, however, that the difference between the sexes is least where *Su* is heterozygous, a condition contrary to that expected if Brink's observations as to the low viability of gametes bearing *su wx* were general.

TABLE 13.—Percentage of waxy seeds on ears not segregating for *Su* (female gametes *Ph 230 L1* and *L2*)

Plant numbers	Total seeds	Number waxy	Per cent waxy	Plant numbers	Total seeds	Number waxy	Per cent waxy
110×3.....	470	236	50.2±1.55	236×28.....	661	336	50.8±1.31
114-a×35.....	174	82	47.1±2.56	602×504.....	773	388	50.2±1.19
119×10.....	1,045	481	46.0±1.07	603×548.....	117	51	43.6±3.08
120×6.....	703	357	50.8±1.27	604×514.....	211	100	47.4±2.32
126×8.....	816	380	46.6±1.17	606×534.....	153	77	50.3±2.72
136×7.....	676	317	46.9±1.29	615×548.....	85	47	55.3±3.64
139×35.....	507	265	52.3±1.49	616×510.....	438	232	53.0±1.61
147×25.....	463	215	46.4±1.56	629×546.....	66	30	45.4±4.13
157×16.....	728	349	47.9±1.25	631×546.....	139	84	60.4±2.81
175×26.....	826	408	49.4±1.17	656×519.....	57	31	54.4±4.45
179×26.....	544	279	51.3±1.44	657×521.....	276	147	53.3±2.02
181×27.....	452	236	52.2±1.58	662×517.....	209	106	50.7±2.33
184×33.....	603	241	40.0±1.34	673×519.....	308	154	50.0±1.92
191×34.....	445	205	46.1±1.59	683×527.....	185	90	48.6±2.48
192×26.....	461	221	47.9±1.57	684×533.....	240	102	42.5±2.15
199×15.....	567	270	47.6±1.41	707×546.....	490	233	47.6±1.52
208×9.....	374	189	50.5±1.74	716×531.....	490	244	50.0±1.52
213×3.....	447	235	52.6±1.59	720×540.....	386	193	50.0±1.72
219×36.....	628	328	52.2±1.35	750×530.....	46	27	58.7±4.92
230×2.....	352	183	52.0±1.79				
232×4.....	306	146	39.9±1.72	Mean.....			48.9±0.37

TABLE 14.—Percentage of waxy seeds in reciprocal crosses involving heterozygous *Su*

Plant numbers	Male gametes			Female gametes			Difference, female-male
	Total seeds	Number waxy	Per cent waxy	Total seeds	Number waxy	Per cent waxy	
1×101.....	995	407	40.9±1.05	727	365	50.2±1.25	9.3±1.63
8×162.....	728	324	44.5±1.24	708	346	48.9±1.27	4.4±1.77
13×149.....	22	8	36.4±1.21	797	381	47.8±1.19	11.4±1.70
20×192.....	270	124	45.9±2.04	711	362	50.9±1.26	5.0±2.40
23×169.....	539	258	47.9±1.45	1,144	556	48.6±.99	7.7±1.76
308×624.....	68	35	51.5±4.08	82	55.4±2.76	3.9±4.92	
513×746.....	394	181	45.9±1.69	49	24	49.0±4.81	3.1±5.10
519×654.....	484	219	45.2±1.54	401	203	50.6±1.68	5.4±2.28
522×644.....	187	88	47.0±2.46	310	158	51.0±1.91	4.0±3.12
535×744.....	86	33	38.4±3.54	194	92	47.4±2.42	9.0±4.28
Mean.....							6.18±.84

TABLE 15.—Percentage of waxy seeds in reciprocal crosses involving homozygous *Su*

Plant numbers	Male gametes			Female gametes			Difference, female-male
	Total seeds	Number waxy	Per cent waxy	Total seeds	Number waxy	Per cent waxy	
3×110.....	781	358	45.8±1.77	470	236	50.2±1.55	4.4±2.35
4×232.....	570	264	46.3±1.41	366	146	39.9±1.72	-6.4±2.22
7×136.....	957	431	45.0±1.09	676	317	46.9±1.30	1.9±1.70
16×157.....	450	167	37.1±1.54	728	349	47.9±1.25	10.8±1.98
35×114.....	402	197	49.0±1.68	174	82	47.1±2.56	-1.9±3.06
510×616.....	829	349	42.1±1.16	438	232	53.0±1.61	10.9±1.98
540×720.....	622	293	47.1±1.35	386	193	50.0±1.71	2.9±2.18
546×707.....	683	290	42.4±1.27	490	233	47.3±1.52	5.1±1.92
548×603.....	801	324	40.4±1.17	117	51	43.6±3.09	3.2±3.31
Mean.....							4.37±1.3

The present material, therefore, offers no support for the hypothesis that male gametes bearing the genes *su* and *wx* are at a disadvantage in effecting fertilization when in competition with gametes bearing other combinations of the genes for sugary and waxy endosperm.

#### POLLEN STORAGE INCREASES THE PROPORTION OF FUNCTIONING WAXY GAMETES

In an attempt to alter the crossover ratio between the aleurone factor *C* and the waxy endosperm factor *Wx*, pollen from plants heterozygous for these linked genes was stored in the collection bags, in the field, for a period approximating seven hours. As a control, approximately one-half of the pollen collected from each plant was applied immediately to the silks of double recessive plants.

Thirteen successful paired pollinations were obtained, and these showed clearly that although the crossover ratio was unaffected (5), the proportion of waxy to nonwaxy gametes was profoundly altered. The functioning waxy-bearing gametes in the stored pollen were in excess of the expected equality and greatly exceeded the proportion of functioning waxy-bearing gametes in the unstored pollen; the latter being less than equality.

Similarly, there was an apparent alteration of the proportion of *C* to *c*-bearing gametes, resulting in an increase in the proportion of white to colored seeds when stored pollen was used. In the cross used, the increase in the proportion of white seeds might be attributed entirely to the linkage between *C* and *Wx*. The parental combinations were *C Wx* and *c wx*, and there was no means of determining whether the observed effect on the ratio of white to colored seeds was the result of linkage with waxy or whether storage actually resulted in a selective action on the *c*-bearing gametes. Accordingly, a somewhat more elaborate experiment was undertaken with a repetition of the original procedure, using in addition a cross in which *C* and *wx* were linked.

All four classes of seed from each cross were planted. The pollen from plants heterozygous for both *C* and *Wx* was used as before in pairs of stored and unstored samples on the double recessive plants. Further, pollen from plants heterozygous for *C* but homozygous for *wx* was applied to plants homozygous for *c* but heterozygous for *Wx*, and the reverse combinations were made also. These latter provide data to measure the effect of storage upon *C* and *Wx* separately.

The same procedure was followed with the cross in which *C* and *wx* were linked.

Considering first the cross in which *C* and *Wx* are linked and where the pollen from the double heterozygote is applied to the double recessive, there are 44 pairs of ears resulting from stored and unstored pollen. The period of storage varied from 6 hours and 10 minutes to 10 hours and 20 minutes, with a mean period of 7 hours and 9 minutes. The ears resulting from stored pollen had on an average 56.8 less seeds than those obtained when the pollen was fresh, which may be taken as a rough approximation of the effect of storage on viability; the period of storage reducing the viability 16.5 per cent.

The data for these 44 pairs are shown in Table 16.

The data presented in Table 16 duplicate, extend, and corroborate those published previously. In measuring the effect of pollen storage

the means and errors are calculated from the arrays of differences between fresh and stored pollen, each difference being weighted by the reciprocal of the squared error of the difference to correct for differences in number of seeds per ear. The procedure followed has been outlined by Collins and Kempton (5, p. 5-6).

Before considering the indicated effect of pollen storage, attention should be directed to the fact that Fisher's (6) test for homogeneity shows that the arrays of differences between the paired samples are not homogeneous, from which it must be concluded that storage is not in itself the cause of the observed differences in gametic proportions. Such a conclusion is not illogical, since a period of storage simply provides an opportunity for unrelated factors such as temperature, light, and humidity to operate; and the effect of such factors might be measured very imperfectly by time of storage.

With this reservation in mind as to the effect of storage the data may be examined. There is a mean increase of  $9.35 \pm 1.26$  per cent of waxy seeds and an increase of  $1.74 \pm 0.67$  per cent of white seeds when stored pollen is applied. Both the white and waxy seeds are below the expected equality in the series of ears resulting from fresh pollen and in excess in the series obtained with stored pollen.

Since white aleurone and waxy endosperm are known to be correlated in this material, owing to the genetic linkage between the *C* and *Wx* genes, any selective agent operating upon one would be expected to affect the other.

In the 44 ears obtained with fresh pollen there is a correlation of  $0.503 \pm 0.76$  between the percentage of white and the percentage of waxy with a regression of white on waxy of 0.272 and of waxy on white of 0.686.

The regressions can be used to predict the change expected in one ratio from a given change in the other. If this be done it is found that increasing the percentage of waxy seeds by the observed amount of 9.35 would be expected to increase the percentage of white seeds by 2.54. The actual increase observed in the percentage of white seeds was  $1.74 \pm 0.67$ , being somewhat, though not significantly, less than the predicted. If the regression of waxy on white be used it is found that having increased the percentage of white seeds by 1.74 an increase of 1.19 in the percentage of waxy would be expected to follow, whereas the increase was 9.35. Clearly the selective action is operative on the gametes bearing the *wx* gene and not on those bearing the aleurone color gene *c*.

As a further measure of the correctness of this conclusion, the regressions of white on waxy and waxy on white may be examined for the array of ears resulting from stored pollen.

If the gametes bearing the waxy gene are operated upon by some selective agent and the departure of the colored-white ratio from equality is due to the linkage between *C* and *Wx*, then the regression of white on waxy should not differ in fresh and stored pollen, but the regression of waxy on white should be much greater in the stored than in the fresh pollen. Both of these conditions are fulfilled. The regression of white on waxy in the stored pollen is found to be 0.276, a very close agreement with the 0.272 found in the fresh pollen, and the regression of waxy on white in the stored pollen is 1.88, whereas in the fresh pollen a regression of but 0.686 was found.

TABLE 16.—Comparison of ears resulting from fresh and stored pollen ( $c \times w \times r \times \frac{1}{2} \times (c \times w \times r \times \frac{1}{2})$ ; the linkage in this comparison is between  $c$  and  $w$  (progeny Dn 416 L3 L1 C5 L4 L1 L1 L25))

Date	Fresh pollen					Stored pollen					Differences			
	Time, a. m.	Fe. male No.	Male No.	Total seeds	Per cent white	Per cent wavy	Per cent over	Time, p. m.	Fe. male No.	Total seeds	Per cent white	Per cent wavy	Per cent over	Time, a. m. - p. m.
Aug. 22	9.00	1584-2	1877	267.46	1.05	50.6	2.06	35.8	4.55	1582	395.50	9.41	70.71	1.51
Aug. 17	9.10	1580	1801	370.48	0.91	75.50	0.41	75.40	4.55	1582	254.50	8.42	72.60	0.22
Do.	9.10	1585	1802	153.49	7.52	72.37	0.22	64.40	5.00	1592	265.47	9.42	72.60	0.22
Aug. 20	7.40	1688	1900-0	66.56	1.4	10.50	1.48	4.4	6.00	1597	104.55	8.43	28.46	3.53
Aug. 22	9.10	1603	1878	240.46	7.4	16.18	3.41	15.48	4.50	1597	194.55	8.43	28.46	3.53
Aug. 16	9.25	1607-2	1888	449.62	0.41	65.45	1.41	64.88	4.4	1612-2	349.50	7.42	60.57	0.21
Aug. 14	10.35	1715	1888	449.62	0.41	54.53	0.41	58.88	4.4	1715	513.48	8.43	70.71	1.51
Aug. 16	7.50	1616	1878	388.42	0.41	69.17	3.41	29.45	4.4	1682	58.52	9.43	70.71	1.51
Aug. 17	9.30	1618-2	1882	148.41	0.41	73.44	0.41	75.87	4.4	1682	60.55	0.43	70.71	1.51
Aug. 16	9.25	1627	1899	162.44	0.41	73.44	0.41	75.87	4.4	1682	240.57	3.42	72.61	0.22
Aug. 17	9.15	1627	1881	59.54	9.41	33.42	4.41	33.34	4.4	1682	400.55	3.42	72.61	0.22
Aug. 19	10.15	1640	1802	433.43	4.41	69.40	0.41	59.38	4.4	1682	197.47	3.42	72.61	0.22
Do.	10.15	1641	1880	381.45	4.41	72.46	4.41	72.42	4.4	1682	244.52	4.41	72.61	0.22
Aug. 17	10.10	1643	1881	368.44	9.41	43.41	0.41	42.36	4.4	1682	244.52	4.41	72.61	0.22
Aug. 15	10.00	1645	1881	553.51	8.41	43.41	0.41	42.36	4.4	1682	316.60	8.42	30.76	0.21
Aug. 16	9.15	1648	1862	100.48	9.42	44.37	4.42	38.37	4.4	1682	316.60	8.42	30.76	0.21
Do.	9.15	1651-2	1894	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Aug. 15	10.05	1651	1882	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Do.	10.05	1656-2	1892	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Aug. 16	9.10	1662	1890	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Aug. 15	10.05	1658-2	1888	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Do.	10.05	1659-2	1886	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Do.	10.05	1660-2	1892	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Do.	10.05	1666	1894	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Do.	10.05	1667	1876	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Aug. 14	10.30	1667	1876	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Aug. 20	7.40	1679	1876	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Aug. 17	9.25	1682	1896	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Aug. 22	9.10	1686	1890	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Aug. 15	10.15	1686	1890	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Aug. 19	10.25	1693	1896	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Do.	10.25	1697-2	1896	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Aug. 15	10.25	1701	1876	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Do.	10.25	1702	1900-0	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Do.	10.25	1704	1900-0	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Do.	10.25	1707	1875	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Aug. 14	10.40	1710-2	1887	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Do.	10.35	1711	1887	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Do.	10.35	1713	1893	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Aug. 16	9.10	1716	1875	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51
Aug. 14	10.35	1717	1900-0	276.52	2.01	37.81	0.41	38.37	4.4	1682	388.50	5.41	70.71	1.51

Aug. 15.....	10.15	1719	1899	609.47	8.1±3.36	47.0±1.36	36.36	5.1±1.31	4.35	1718	229.59	0.4±2.19	56.8±2.20	20.36	0.4±2.14	11.7±2.58	9.8±2.59	-5.2±2.51	6.20	380
Aug. 16.....	9.10	1721	1900-C	59.52	7.4±4.38	40.70	7.4±3.30	27.0±3.88	4.45	1720	145.40	0.2±2.80	68.8±3.2	60.29	3.2±2.64	-3.5±5.19	27.6±3.02	2.3±1.64	7.36	-86
Do.....	9.10	1723	1878	229.19	8.1±2.24	46.6±2.24	6.2±2.22	6.2±2.22	4.45	1722	360.51	1.1±1.93	49.8±1.93	31.1	3.1±1.78	-11.3±2.95	-11.3±2.95	-11.3±2.95	7.35	-82
Aug. 17.....	9.25	1724	1878	369.45	4.1±1.75	47.47	3.1±1.75	38.8±1.71	5.05	1721-2	360.43	0.4±1.70	46.8±1.70	42.9	4.2±1.75	-1.5±2.48	-2.0±2.48	4.2±2.44	7.40	6
Mean.....	.....	.....	.....	345.40	0.4±.53	44.1±.78	38.8±.55	.....	.....	.....	289.50	0.6±.38	53.7±.94	37.4±.38	1.74±.67	.....	9.35±1.26	-2.0±1.52	7.00	56

\* Time is given in hours and minutes.

TABLE 17.—Comparison of ears resulting from fresh and stored pollen ( $c \times wx \times r \times \delta \times C \times W \times wx \delta$ ; the linkage in this comparison is between  $C$  and  $wx$  (progeny  $Dh \ 416 \ L3 \ L1 \ C5 \ L4 \ L1 \ L2 \ L25$ )

Date	Fresh pollen					Stored pollen					Differences					Time, p. m. a. m.	Seeds, a. m. p. m.
	Time, a. m.	Fe- male No.	Male No.	Total seeds	Per cent white	Per cent waxy	Per cent crossing over	Time, p. m.	Fe- male No.	Total seeds	Per cent white	Per cent waxy	Per cent crossing over	Per cent waxy, p. m.-a. m.	Per cent crossing over, p. m.-a. m.		
Aug. 19.....	10.00	1583	2108	225.50	7.1±2.24	43.3±2.23	7.1±2.18	5.00	1583-2	295.48	8.1±1.90	51.1±1.90	56.29	2.1±1.78	-1.9±2.98	7.00	-70
Do.....	10.00	1608-2	2118	297.51	2.1±1.94	47.8±1.95	8.1±1.83	3.00	1603	309.51	1.1±1.92	12.7±1.90	50.36	5.1±1.83	3.7±2.81	7.00	-12
Aug. 15.....	10.00	1614-2	2090	417.52	0.1±1.65	46.3±1.64	3.1±1.53	3.00	1614	291.52	8.4±2.07	14.4±2.07	50.31	4.1±1.83	0.0±2.46	7.00	156
Aug. 15.....	10.35	1624-2	2092	422.51	0.1±1.64	46.3±1.64	3.1±1.53	4.30	1621	345.43	8.1±1.80	15.5±1.80	50.31	3.1±1.83	1.7±2.22	7.00	77
Aug. 14.....	10.20	1625	2093	669.40	6.1±3.30	46.3±3.30	0.1±1.49	4.50	1676	433.52	5.1±1.58	49.1±1.58	51.1	3.1±1.83	0.4±2.05	6.30	212
Do.....	10.20	1642	2093	380.50	8.1±1.71	43.3±1.69	3.1±1.59	5.00	1642-2	914.5	0.4±3.50	50.2±7.3	52.35	3.1±1.83	4.0±3.50	6.30	236
Do.....	10.25	1649-2	2092	377.56	8.1±5.30	40.4±5.30	4.0±2.45	4.55	1649	330.50	0.1±1.45	44.1±1.44	50.36	5.1±1.83	1.8±2.92	6.30	499
Aug. 15.....	10.35	1665-2	2117	178.50	0.1±2.52	49.4±2.52	7.1±2.30	4.35	1669	428.46	8.1±1.63	50.1±1.63	50.36	0.1±1.83	1.7±2.27	7.00	-110
Aug. 16.....	9.45	1905	2103	710.51	0.1±1.90	48.3±1.91	0.1±1.71	4.45	1900-2	518.51	4.1±1.48	48.3±1.48	50.36	0.1±1.83	0.0±1.78	6.20	272
Aug. 15.....	10.30	1906	2106	163.52	1.2±2.64	43.3±2.64	0.1±2.58	5.10	1912	424.31	4.1±1.51	47.4±1.51	57.28	0.1±1.40	1.2±2.98	7.15	-261
Aug. 17.....	9.55	1913	2110	127.59	0.1±2.94	43.3±2.94	0.1±2.63	5.10	1914	460.51	1.1±1.57	48.3±1.57	57.28	0.1±1.40	1.2±2.98	7.15	-333
Do.....	10.00	1914-2	2108	117.52	1.1±3.12	42.7±3.12	0.1±2.63	5.10	1915	114.52	6.1±3.14	50.36	1.5±3.12	0.1±1.40	1.2±2.98	7.15	-3
Aug. 14.....	11.10	1917	2113	370.55	1.1±3.12	42.7±3.12	0.1±2.63	5.10	1917-2	220.36	7.1±2.14	50.36	2.1±2.84	0.1±1.40	1.2±2.98	7.15	250
Aug. 17.....	9.55	1919-2	2094	269.50	2.1±1.97	43.3±1.97	0.1±1.86	5.15	1919	182.53	3.2±2.48	55.2±2.48	57.28	0.1±1.40	1.2±2.98	7.15	33
Do.....	9.50	1926	2117	149.41	0.1±2.72	47.4±2.72	0.1±2.63	5.15	1922	182.53	3.2±2.48	55.2±2.48	57.28	0.1±1.40	1.2±2.98	7.15	61
Aug. 14.....	11.15	1923	2088	265.54	7.1±1.98	45.6±1.98	3.1±1.68	5.15	1923	300.44	1.1±1.90	49.1±1.90	50.36	0.1±1.83	1.7±2.27	7.00	-33
Aug. 17.....	9.50	1929	2118	384.40	4.1±1.77	42.8±1.77	0.1±1.68	5.15	1928	263.41	1.1±2.01	49.1±1.90	50.36	0.1±1.83	1.7±2.27	7.00	-297
Aug. 19.....	10.00	1941	2110	158.53	8.1±2.66	44.3±2.66	0.1±1.68	5.15	1928-2	300.44	1.1±2.01	49.1±1.90	50.36	0.1±1.83	1.7±2.27	7.00	-33
Aug. 19.....	10.00	1942	2095	189.50	8.1±2.66	44.3±2.66	0.1±1.68	5.15	1928-2	300.44	1.1±2.01	49.1±1.90	50.36	0.1±1.83	1.7±2.27	7.00	-33
Aug. 22.....	10.00	1943	2106	469.52	4.1±1.55	45.6±1.55	0.1±1.68	5.15	1928-2	300.44	1.1±2.01	49.1±1.90	50.36	0.1±1.83	1.7±2.27	7.00	-33
Aug. 15.....	10.30	1936	2106	230.55	2.1±1.67	42.7±1.67	0.1±1.68	5.15	1936-2	448.54	7.1±2.76	50.36	1.1±2.76	0.1±1.40	1.2±2.98	7.15	105
Aug. 19.....	10.00	1943-2	2091	230.55	2.1±1.67	42.7±1.67	0.1±1.68	5.15	1936-2	448.54	7.1±2.76	50.36	1.1±2.76	0.1±1.40	1.2±2.98	7.15	105
Do.....	10.00	1951	2094	313.56	5.1±1.89	42.8±1.89	0.1±1.68	5.15	1951-2	308.57	8.1±1.89	48.3±1.89	51.1	0.1±1.83	1.7±2.27	7.00	-19
Mean.....	.....	.....	.....	302.52	1.1±.37	46.1±.47	0.1±.44	.....	.....	319.48	3.1±.87	51.1±.90	53.8±.91	.....	1.7±.80	6.52	-17

\* Time is given in hours and minutes.

If the conclusion be correct that the proportion of gametes bearing the *c* gene to those bearing its dominant allelomorph *C* is altered by pollen storage only as the result of this gene being linked with *Wx*, then it must follow that when the linkage is reversed (*C* being associated with *wx*) an increase in the percentage of waxy seeds must be accompanied by a decrease in the percentage of white seeds.

To determine the correctness of this assumption the data shown in Table 17 have been compiled. The ears shown in this table were obtained by the same procedure as was followed where the linked factors were *C Wx*, and the biometrical constants have been calculated as described for the 44 pairs of pollinations shown in Table 16. There is a slight difference between the two sets of data in that the period of storage was less for the ears shown in Table 17 than for those in Table 16. This difference in time was unavoidable since the progeny that produced the 44 pairs of ears was pollinated first and the time that elapsed in handling this progeny necessarily was at the expense of the succeeding progenies. Since the 44 pairs were a direct repetition of the original material the emphasis was placed on obtaining adequate data from this progeny first. The mean time of storage for the ears shown in Table 17 was 6 hours and 52 minutes as compared with a period of 7 hours and 9 minutes for the ears in Table 16.

Using the mean difference in number of seeds produced by stored pollen as compared with fresh pollen as a measure of the effect of storage on vitality, it is seen that the stored pollen on an average produced approximately 17 more seeds per ear (actually 17.04 seeds) than the fresh pollen. However, the factors other than storage affecting the number of seeds are so numerous that it is extremely questionable, with only 24 pairs, whether the difference in number of seeds can be utilized as an indication of the condition of the pollen. The observed difference indicates an increase in viability of 5.6 per cent.

It is seen that the percentage of waxy seeds is increased in those ears resulting from the application of stored pollen when compared with ears resulting from the duplicate samples of fresh pollen. The difference is  $6.14 \pm 1.07$  per cent and may be compared with that of  $9.35 \pm 1.26$  found in the series shown in Table 16. Although the ears resulting from pollen stored, on an average, 6 hours and 52 minutes have a lower percentage than those obtained with pollen stored for 7 hours and 9 minutes the difference of  $3.21 \pm 1.65$  per cent between the two sets can be attributed to chance, making it unnecessary to assume that a difference of 17 minutes in the period of storage results in such a large alteration in the proportion of waxy-to-horny-bearing gametes.

Having seen that the effect of storage on the proportion of functioning waxy gametes is similar for the two series, the effect on the percentage of white may be examined. The data show that in this progeny where waxy endosperm and colored aleurone are associated in inheritance the percentage of white seeds resulting from the use of stored pollen is less than that from fresh pollen. This condition is in accord with the hypothesis that whatever selective force is operative on the stored gametes effects directly only the gametes bearing the waxy gene. In this case the change produced in the proportion of white to colored seeds is but a secondary effect due to the genetic correlation of waxy with the factor for colored aleurone.

The change observed in the percentage of white is significant statistically, and in amount accords fairly well with the percentage expected from the regression of white on waxy. Thus the regression of white on waxy in the fresh pollen was  $-0.305$  and in the stored pollen  $-0.155$ . Using the figure for the fresh pollen as a constant from which to calculate the percentage of white seeds expected to follow a change of 6.14 per cent in the waxy, it is found that the percentage of white seeds in the stored pollen should be 1.87 less than that in the fresh pollen, whereas it was  $3.43 \pm 0.89$  per cent less. The difference, however, though large is not significant.

#### EFFECT OF POLLEN STORAGE WHERE ONLY $Wx$ IS HETEROZYGOUS

There are 24 pairs of ears that resulted from crosses where plants heterozygous for  $Wx$  and homozygous for  $c$  were used as male parents on plants homozygous for  $wx$  and heterozygous for  $C$ . In these cases a period of storage can affect only the proportion of waxy to nonwaxy since the male gametes were all of one kind with respect to color, namely, homozygous for  $c$ . The data for these ears are shown in Table 18. The plants bearing these ears were sibs of the plants producing the 44 pairs shown in Table 16.

The mean period of storage for the pollen used on 24 pairs of ears for which data are given in Table 18 was 6 hours and 20 minutes and the ears from stored pollen had on an average 34.1 less seeds than those from fresh pollen, an indicated death rate of 10.2 per cent.

As in the previous two cases, the percentage of waxy seeds on ears resulting from stored pollen is greater than on ears produced from fresh pollen, the excess in this instance being  $7.13 \pm 2.48$  per cent. No difference is expected in the ratio of white to colored seeds and none was found.

A similar set of pollinations was made in the progeny where  $C$  and  $wx$  were linked and 10 successful pairs were obtained. The plants from which these ears came were sibs of the plants shown in Table 17, but differed from them in that the female parents were constituted  $Cc\ wx\ wx$  and the male parents had the gametic constitution  $c\ c\ Wx\ wx$ . In these cases, therefore, the storage of pollen could affect only the proportion of waxy to nonwaxy, and the percentage of white seeds should be alike on the ears from fresh and stored pollen.

The data are shown in Table 19.

In full agreement with expectation there was no difference between the ears resulting from fresh pollen and those obtained with stored pollen in the percentage of white seeds; the difference being  $0.76 \pm 0.85$  per cent.

In the case of the waxy-nonwaxy ratio, however, it is seen that the ears resulting from stored pollen have on an average  $4.30 \pm 1.47$  per cent more waxy seeds than those obtained with fresh pollen.

With these ears the period of storage averaged six hours and six minutes, the shortest period for any of the four groups studied, and the effect on the waxy ratio is less than in the other three groups. The ears resulting from stored pollen had on an average 126 seeds less than those obtained with fresh pollen, indicating a decrease in viability of the stored gametes, of 28 per cent.

TABLE 18.—Comparison of ears resulting from fresh and stored pollen ( $C \times wx \times wx \times cc \times Wz \times vx \times \delta$ ); no linkage is involved in these ears (progeny Dh 416 L3 L1 C5 L4 L1 L1 L25)

Fresh pollen				Stored pollen				Differences									
Time, a. m.	Fe- male No.	Male No.	Total seeds	Per cent white	Per cent waxy	Per cent crossing over <sup>a</sup>	Time, p. m.	Fe- male No.	Total seeds	Per cent white	Per cent waxy	Per cent crossing over	Per cent white, p. m.-a. m.	Per cent waxy, p. m.-a. m.	Per cent crossing over, p. m.-a. m.	Time, <sup>b</sup> p. m.- a. m.	Seeds, a. m.- p. m.
9. 15	1743	1821	33547	2.2±1.83	54.0±2.66	44.2±1.53	5.90	1743-2	59580	3.3±4.31	64.0±1.03	51.1±4.36	12.1±4.08	40.9±3.28	9.9±4.73	7.45	276
11. 00	1745	1813	56585	9.4±4.42	35.7±5.60	40.9±5.76	3.05	1745-2	31051	3.1±1.91	44.5±1.00	48.5±1.90	-7.6±4.82	8.8±5.92	5.7±6.06	7.45	-254
11. 15	1746	1801	50151	9.1±4.50	46.9±1.49	52.8±1.49	4.40	1746-2	43651	1.1±1.60	48.4±1.61	48.5±1.61	-8.2±2.19	9.8±2.19	6.05	65	
9. 35	1747	1827	49053	3.1±1.51	44.7±1.51	46.5±1.52	5.05	1760	18061	1.7±7.75	77.7±8.2	08.00±7.78	7.8±2.57	33.9±2.57	13.5±7.92	7.30	472
9. 35	1770	1814	15451	3.2±2.72	49.4±2.72	51.4±2.72	4.35	1749	26149	0.2±2.08	57.8±2.08	46.9±2.08	-2.3±3.42	8.3±3.42	4.4±5.40	6.30	-107
11. 15	1748	1810	57349	4.1±1.40	46.6±1.40	48.2±1.40	4.35	1749	51951	0.2±1.48	52.8±1.48	51.3±1.48	1.6±2.05	5.2±2.05	5.20	54	
10. 35	1750	1833	11048	2.3±2.21	40.9±3.16	49.9±3.21	4.35	1750-2	34051	5.1±1.82	58.8±1.79	46.4±1.82	3.3±3.63	17.9±3.63	3.80	-280	
11. 30	1751	1812	51149	9.1±1.49	45.1±1.49	49.4±1.49	4.40	1751-2	40050	0.4±7.04	74.4±1.51	57.4±1.57	5.6±4.84	19.1±2.65	10.8±4.90	7.05	451
9. 35	1753-2	1837	50245	4.1±1.49	45.6±1.49	47.6±1.50	4.40	1753-2	5151	0.4±7.04	74.4±1.51	57.4±1.57	5.6±4.84	19.1±2.65	10.8±4.90	7.05	451
11. 20	1758	1843	29848	1.2±0.90	50.4±2.02	52.7±2.09	4.35	1758-2	43340	0.2±1.61	49.4±1.61	49.4±1.61	9.2±2.65	19.1±2.65	10.8±4.90	7.05	451
10. 45	1789	1845	62746	5.1±1.34	42.1±1.33	51.8±1.34	4.35	1771	52049	2.1±1.47	45.0±1.47	49.4±1.48	-3.2±2.00	2.0±2.00	1.9±2.00	6.20	107
11. 40	1767	1865	44762	1.1±1.59	44.8±1.58	48.3±1.59	4.45	1767-2	25755	6.2±2.08	49.0±2.10	47.7±2.09	3.5±2.51	2.6±2.51	2.2±2.51	7.05	-1
10. 35	1769-2	1810	8255	5.3±3.08	53.8±3.03	55.0±3.09	5.10	1770	41850	7.1±1.50	52.9±1.50	49.4±1.50	-1.4±2.26	8.9±2.26	-8.2±3.02	6.33	319
11. 00	1772	1837	11757	3.3±3.03	53.8±3.03	55.0±3.09	5.05	1772-2	45347	5.1±1.57	45.0±1.57	52.1±1.58	-0.8±3.46	7.3±3.46	7.2±3.46	7.05	-365
9. 20	1773	1840	26952	4.2±2.04	42.8±2.03	43.2±2.03	4.30	1772-2	36647	8.3±3.70	50.8±1.70	48.4±1.70	3.4±2.88	10.3±2.88	5.2±2.70	6.33	-97
10. 35	1774	1842	5347	2.4±4.60	47.2±4.60	48.2±4.63	4.30	1782-2	28540	6.3±3.06	49.3±4.36	48.3±4.36	-2.1±2.06	6.3±2.06	1.8±2.06	6.33	-32
11. 25	1775	1808	42646	5.1±1.63	46.6±1.62	46.6±1.63	4.35	1775-2	31850	0.1±1.68	48.4±1.68	48.4±1.68	2.4±2.12	3.8±2.12	2.0±2.12	5.10	171
9. 45	1779	1831	63847	1.1±1.31	47.1±1.31	50.6±1.31	4.35	1781	41530	1.1±1.66	48.4±1.66	48.4±1.66	2.4±2.12	3.8±2.12	2.0±2.12	5.10	247
11. 30	1780	1831	63847	7.1±3.47	41.4±1.31	50.6±1.31	4.35	1781-2	22500	1.1±1.78	48.4±1.78	48.4±1.78	8.1±4.08	21.4±4.08	8.6±3.39	6.15	163
10. 35	1782-2	1838	18543	4.2±2.46	40.0±2.41	42.7±2.45	5.10	1783-2	6456	2.4±1.92	28.1±3.75	37.9±4.08	2.7±3.38	5.0±3.38	6.6±3.39	6.20	-57
10. 45	1786	1841	32048	1.1±1.88	49.7±1.88	46.6±1.88	5.05	1786-2	22948	0.2±2.22	48.4±2.22	48.4±2.22	3.5±2.98	9.3±2.98	4.2±3.00	7.25	-234
10. 50	1788	1814	17245	3.2±2.53	43.0±2.53	43.9±2.53	5.05	1788-2	41952	5.1±1.64	50.6±1.65	50.6±1.65	.49±.56	7.13±2.48	.005±.61	6.20	34
9. 40	1790-2	1802	18147	0.2±2.50	49.7±2.50	46.4±2.50	5.05	1790	29950	2.2±2.00	51.5±3.63	49.2±2.32	.49±.56	7.13±2.48	.005±.61	6.20	34
Date			Mean														

<sup>a</sup> No crossing over is possible in crosses of this nature. This column gives the percentage of crossover as calculated from the four classes of seed.<sup>b</sup> Time is given in hours and minutes.

TABLE 19.—Comparison of ears resulting from fresh and stored pollen ( $C \times wx \times wx \times c \times c \times Wx \times wx \times \delta$ ), no linkage is involved in these ears (progeny Dh 416 L3 L1 C5 L4 L1 L2 L25)

Date	Fresh pollen					Stored pollen					Differences					
	Time, a. m.	Fe- male No	Male No	Total seeds	Pct white	Pct crossing over <sup>a</sup>	Time, p. m.	Fe- male No	Total seeds	Pct white	Pct crossing over	Pct crossing over, p. m. - a. m.	Pct way, p. m. - a. m.	Time <sup>b</sup> , p. m. - a. m.	Seeds, a. m. - p. m.	
Aug. 15	11 00	1908	2008	134 42	5±2	87.46	3±2	90.44	8±2	89	4 50	1908-2	450	1908-2	4 50	326
Aug. 19	10 45	1981	2022	306 46	5±1	68.44	7±1	68.40	8±1	69	5 20	1909	5 20	1909	5 50	67
Aug. 16	8 45	1983	2044	373 45	7±1	52.44	4±1	73.39	1±1	70	5 00	1974	3 3±2 50	1 0±2 31	7 35	13
Aug. 15	10 30	1975	2016	593 48	8±1	40.43	2±1	45.51	1±1	47	1 50	1985	21.0±2 12	1.0±2 33	6 00	121
Aug. 14	11 30	1977	2016	369 52	6±1	75.43	0±1	74.50	4±1	75	5 25	1977-2	3.0±2 21	3.0±2 24	5 50	325
Aug. 16	9 50	1987	2012	374 52	5±2	60.46	5±2	10.46	8±2	10	4 55	1984	3.8±3 36	3.8±3 36	7 00	211
Aug. 14	11 30	1986	2039	637 48	0±1	30.42	7±1	30.45	9±1	33	5 20	1980-2	2.5±2 38	11.9±3 70	5 50	544
Aug. 15	10 50	1989	2041	840 50	8±1	10.47	3±1	16.49	8±1	16	4 50	1987	1.5±1 97	3.2±2 98	6 00	399
Aug. 14	11 30	1988	2041	398 47	0±1	08.45	0±1	08.45	0±1	08	5 30	1988-2	3.2±2 48	3.2±2 48	5 50	56
Aug. 15	10 45	1991	2048	570 52	8±1	40.46	3±1	40.50	0±1	41	4 50	1990-2	3.2±2 47	3.2±2 48	6 05	300
Mean				450 49	3±	.03	45 0±	.39	17.8±	.76			4.3±1 47	2.2±	0 00	126

<sup>a</sup> No crossing over is possible in crosses of this nature. This column gives the percentage of crosses as calculated from the four classes of seeds.<sup>b</sup> Time is given in hours and minutes.

There can be little doubt that the storage of pollen, heterozygous for *Wx*, for periods of several hours, increases the proportion of functioning gametes bearing the *wx* gene.

As a means of determining how the varying periods of time used, affected the percentage of waxy seeds obtained, the correlation of time and percentage may be examined. The coefficient for the 68 sister ears shown in Tables 16 and 18 is found to be 0.325. This is not a very close relationship, but the regression of percentage on time is found to be 4.04. Since percentage distributions are being dealt with the regressions certainly are not linear but as a very general indication of the effect of time the regression will serve. It is apparent however, that on this basis, if the pollen were stored for 18 hours, all the resulting seeds would be waxy.

A very similar measure of the same thing is obtained with the coefficient of correlation between time and the magnitude of the difference between fresh and stored pollen. Here the coefficient is 0.346 and the regression of difference on time is 5.37, indicating that with each change of one hour in period of storage there is a corresponding change of 5 per cent in the difference between fresh and stored pollen.

#### POLLEN STORAGE INCREASES THE PROPORTION OF GAMETES BEARING THE ALEURONE COLOR FACTOR *c*

Having seen that the proportion of white to colored seeds differs in stored and fresh pollen when the waxy gene is involved, and, further, having indirect evidence that the disturbance is due to the effect of storage on the gametes bearing the *wx* gene, it may be well to examine the data accumulated from sister plants where only the *Cc* gene is involved.

These data consist of ears resulting from crosses of the nature *c c Wx wx* ♀ × *C c wx wx* ♂ and were obtained from two progenies. In crosses of this nature four classes of seeds are obtained, but since the male gametes were all of one kind with respect to waxy, namely, *wx*, there can be no differential effect of storage operating upon this gene. The results are shown in Tables 20 and 21.

The percentage of waxy should be alike in both morning and afternoon pollinations, and such is the case. It is of interest to observe that the percentage very closely approximates the expected 50 in both arrays.

The difference in percentage of white in Table 20 is  $1.82 \pm 0.57$ , and for the ears in Table 21 is  $1.87 \pm 0.74$ . In neither case is the difference significant in comparison with its probable error, but in both instances the percentage of white seeds is higher on those ears resulting from stored pollen. Combining the two progenies, the mean difference becomes  $1.84 \pm 0.29$ , clearly a significant difference, showing that the storage of pollen tends to increase the proportion of functioning gametes bearing the *c* aleurone factor.

The observed changes in the proportions of white and colored seeds resulting from stored pollen were attributed entirely to the linkage between the genes *c* and *wx*. This conclusion seemed justified since the actual percentage of white seeds agreed closely with the percentage predicted from the regressions of white on waxy.

TABLE 20.—Comparison of ears resulting from fresh and from stored pollen ( $G \times Wz$  vs  $Q \times C$   $u \times w \times \delta$ ); no linkage is involved in these ears (progeny Dh 416 L3 L1 C5 L4 L1 L1 L25)

Fresh pollen				Stored pollen				Differences									
Date	Time, a. m.	Fe-male No.	Male No.	Total seeds	Per cent white	Per cent waxy	Per cent crossing over	Time, p. m.	Fe-male No.	Total seeds	Per cent white	Per cent waxy	Per cent crossing over	Per cent waxy, p. m.-a. m.	Per cent crossing over, p. m.-a. m.	Time, p. m.-a. m.	Seeds, a. m.-p. m.
Aug. 15	11.15	1801	1746	514.48	0±1.48	50.4±1.48	4±1.48	1801-2	4.40	386.48	2±1.71	51.3±1.71	52.3±1.70	0.9±2.26	2.9±2.25	5.25	128
Aug. 16	11.30	1802	1771	569.50	3±1.41	48.8±1.41	47.5±1.42	1802-2	4.45	367.50	8±1.43	44.8±1.43	42.3±1.43	6.0±2.40	5.2±2.02	5.15	12
Aug. 16	9.40	1803	1757	379.48	0±1.73	51.4±1.73	9±1.73	1803-2	4.35	557.51	2±1.77	74.6±1.77	72.5±1.76	1.3±2.47	6.2±2.47	5.15	18
Aug. 17	9.45	1804	1742	159.40	0±2.07	49.0±2.07	8±2.08	1804-2	5.10	174.70	7±2.32	41.4±2.32	50.5±2.33	7.6±3.66	9.1±3.68	7.05	18
Aug. 16	9.35	1805-2	1751	324.48	4±1.87	50.0±1.87	3±1.88	1805-2	4.45	351.50	4±1.79	49.0±1.79	52.2±1.79	1.6±2.50	1.9±2.50	7.25	15
Aug. 17	11.25	1808	1775	323.48	9±1.87	48.9±1.87	53.9±1.88	1808-2	4.45	310.48	7±1.80	49.0±1.80	50.6±1.89	2.0±2.66	1.9±2.66	7.10	27
Aug. 15	10.40	1809	1770	63.47	6±4.23	50.8±4.23	6±4.23	1814	4.45	484.46	6±1.53	47.4±1.53	46.9±1.53	2.9±4.51	7.3±4.50	6.30	7
Aug. 19	11.15	1810	1748	342.52	0±1.82	50.3±1.82	4±1.82	1812	4.45	511.52	4±1.48	51.0±1.48	50.9±1.49	6.2±3.35	3.5±2.35	5.30	421
Aug. 15	11.30	1812	1751	644.52	8±1.70	47.4±1.70	46.6±1.71	1812-2	4.45	363.40	8±1.33	48.8±1.33	50.2±1.33	13.7±5.74	12.6±5.75	5.15	169
Aug. 14	11.05	1813	1745	280.40	3±2.52	3±1.32	51.8±1.34	1813-2	5.05	181.57	1±8.90	42.8±8.90	25.7±8.91	8.3±9.11	1.6±2.89	6.00	1
Aug. 19	10.40	1814-2	1782	241.44	9±2.15	50.0±2.15	10±2.16	1820-2	5.10	48.39	0±4.75	50.4±4.75	43.4±4.82	3.8±9.11	2.8±7.52	6.30	296
Aug. 22	10.50	1820-2	1741	461.47	9±2.15	50.0±2.15	10±2.16	1820-2	5.10	327.52	0±4.75	50.4±4.75	43.4±4.82	1.3±2.43	2.9±2.48	7.30	193
Aug. 17	9.35	1828	1753	205.40	0±2.67	54.3±2.67	6±2.67	1830-2	5.00	384.50	3±1.72	51.3±1.72	51.3±1.72	1.3±2.68	2.9±2.68	7.20	134
Aug. 17	9.35	1829	1782	403.46	0±1.67	54.3±1.67	6±2.67	1830-2	5.00	119.52	3±3.09	47.3±3.09	47.3±3.09	1.2±2.69	2.9±2.69	7.25	284
Aug. 15	11.30	1831	1780	220.45	0±2.20	46.8±2.20	47.4±2.20	1830-2	4.45	336.45	8±1.83	49.0±1.83	48.5±1.83	2.9±2.91	1.0±2.91	5.15	116
Aug. 15	11.30	1834	1781	623.40	0±1.35	50.2±1.35	51.1±1.35	1834-2	4.45	177.52	0±2.52	53.3±2.52	49.8±2.53	2.9±2.86	2.1±2.87	5.20	416
Do.	11.00	1837	1772	689.50	2±1.28	47.0±1.28	49.0±1.28	1837-2	5.10	319.53	0±2.52	53.3±2.52	49.8±2.53	5.4±2.28	2.1±2.27	6.10	370
Aug. 14	10.30	1838	1785	225.53	3±2.23	48.9±2.23	24.48	1838-2	5.10	148.56	1±2.74	47.3±2.74	47.3±2.74	1.6±3.55	1.3±3.56	7.15	77
Aug. 16	9.30	1839	1786	725.40	1±1.25	51.1±1.25	46.8±1.24	1841-2	5.10	209.47	0±2.85	48.8±2.85	47.3±2.85	3.4±3.11	7.3±3.10	6.30	586
Aug. 14	10.50	1841	1786	331.48	0±1.88	49.0±1.88	47.3±1.89	1841-2	5.10	239.47	0±2.85	48.8±2.85	47.3±2.85	1.7±2.77	10.7±2.77	6.20	32
Aug. 19	10.40	1842	1783	278.54	7±2.01	45.7±2.01	50.4±2.02	1842-2	4.50	247.55	0±2.85	48.8±2.85	47.3±2.85	4.5±2.94	7.2±2.95	6.30	31
Aug. 15	11.20	1843	1788	336.46	1±1.83	47.6±1.83	47.6±1.83	1843-2	5.10	262.49	2±2.08	50.2±2.08	47.3±2.08	5.4±2.77	1.9±2.77	6.25	74
Aug. 14	10.45	1844	1788	576.48	8±1.40	51.9±1.40	53.4±1.40	1844-2	5.15	622.46	0±1.35	50.3±1.35	48.1±1.35	1.6±1.94	5.3±1.94	6.35	46
Do.	10.40	1845	1789					1815-2									
Mean				300.40	3±.20	49.9±.20	49.9±.35			302.51	0±.57	40.9±.57	39.50±.48	.20±.56	.54±.61	6.11	88

<sup>a</sup> In crosses of this nature no crossing-over is possible. This column gives the percentage of crossovers as calculated from the four classes of seeds.<sup>b</sup> Time is given in hours and minutes.

TABLE 21.—Comparison of ears resulting from fresh and stored pollen ( $c \times Wx vx \varnothing \times C \times vx vx \delta$ ); no linkage is involved in these ears (progeny Dh 416 L3 L1 C5 L4 L1 L2 L25)

Date	Fresh pollen					Stored pollen					Differences			
	Time, a. m.	Fe- male No.	Male No.	Total seeds	Per cent white	Per cent waxy	Per cent crossing over <sup>a</sup>	Time, p. m.	Fe- male No.	Total seeds	Per cent white	Per cent waxy	Per cent crossing over	Time, <sup>b</sup> Seeds, p. m.-a. m.; p. m.
Aug. 17	10.00	2001	1970	34240.1±1	82.55.0±1.80	51.6±1.83	5.15	2006-2	24151	4±2.2	16.40.4±2	10.46.8±2.16	2.3±2.82	7.15
Aug. 15	11.00	2004	1970	11046.4±3	19.42.7±3.18	48.5±3.21	5.00	2004-2	11152.2±3.19	52.2±3.19	48.8±3.20	5.1±2.83	-5.1±2.83	6.00
Do.	11.00	2006	1976	15742.7±2	66.51.6±2.70	50.5±2.69	4.55	2019	24440.6±2	15.48.0±2.16	46.1±2.15	0.9±3.51	9.5±3.52	3.35
Aug. 19	10.50	2003-2	1981	24951.0±2	14.51.2±2.12	47.8±2.13	5.30	2007	22744.0±2	22.47.1±2.23	40.0±2.24	-7.0±3.08	-3.0±3.44	6.40
Aug. 15	11.00	2008	1968	63347.4±1	33.40.1±1.34	52.7±1.33	5.00	2008-2	56849.7±1	38.44.5±1.37	52.9±1.37	1.2±3.08	1.2±3.09	6.00
Do.	10.55	2016	1985	64160.1±1	32.48.6±1.33	47.6±1.32	4.55	2016-2	52448.7±1	47.49.0±1.47	49.1±1.47	-1.4±1.98	2.0±1.97	6.00
Do.	11.00	2017	1974	23142.4±2	18.51.9±2.22	52.7±2.20	4.55	2019-2	62251.4±1	35.46.6±1.35	49.9±1.35	9.0±2.86	-3.1±2.88	5.55
Do.	10.50	2021	1987	68750.4±1	28.51.7±1.28	48.9±1.28	4.55	2021-2	67556.4±1	29.49.0±1.29	50.9±1.30	6.0±1.82	-2.7±1.82	6.05
Aug. 17	10.00	2022	1974	16543.6±2	60.46.7±2.61	49.3±2.62	5.15	2022-2	180558.2±2	46.51.1±2.47	48.3±2.46	9.6±3.98	-1.0±3.00	7.15
Aug. 16	9.50	2024	1974	9742.8±3	38.45.4±3.41	38.0±3.32	4.55	2035	41250.7±1	66.45.9±1.65	53.3±2.46	8.4±3.76	15.5±3.07	7.05
Aug. 17	10.05	2029	1983	23446.6±2	19.48.7±2.20	46.5±2.19	5.15	2032	41951.1±1	65.54.4±1.64	51.7±1.65	4.5±2.74	5.2±2.74	7.10
Aug. 15	10.50	2039	1989	59146.2±1	38.53.1±1.38	52.0±1.38	4.50	2034	27354.6±2	03.49.8±2.04	48.3±2.04	8.4±2.46	-3.7±2.46	6.00
Aug. 16	10.50	2036	1985	9150.5±3	34.46.2±3.35	47.3±3.52	4.55	2042	24351.0±2	16.45.7±2.15	47.8±2.16	-5.3±3.98	5.3±3.98	7.05
Aug. 14	11.25	2041	1988	57654.5±1	43.54.5±1.40	52.7±1.40	5.30	2041-2	32249.1±1	87.49.7±1.87	50.8±1.88	2.4±2.27	-2.4±2.34	6.05
Aug. 15	10.45	2048	1991	79640.0±1	23.49.3±1.24	47.8±1.24	4.50	2043	31151.4±1	91.43.4±1.89	53.7±1.90	5.1±1.96	-5.9±2.26	7.05
Aug. 19	10.50	2058	1983	55650.9±1	43.50.2±1.43	53.6±1.45	5.30	2053-2	62351.4±1	35.48.2±1.35	52.8±1.35	6.1±1.98	8±1.98	6.40
Aug. 15	11.10	2055	1986	64040.2±1	33.51.6±1.33	48.6±1.33	5.00	2055-2	10444.2±3	71.57.7±1.26	52.9±3.30	4.3±3.56	3.3±3.56	5.30
Do.	11.10	2056	1984	42853.5±1	62.45.6±1.62	50.3±1.63	5.00	2058	43948.5±1	61.51.7±1.60	50.4±1.61	-5.0±2.28	6.1±2.28	5.30
Do.	11.10	2062	1983	25251.2±2	12.48.4±2.12	46.4±2.12	5.05	2062-2	33750.4±1	83.90.7±1.83	47.4±1.83	-8±2.80	1.0±2.80	5.55
Mean				39040.2±	48.50.4±	44.49.9±			36450.9±	43.48.6±	47.50.4±	1.87±.74	-1.32±.64	6.19

<sup>a</sup> No crossing over is possible in crosses of this nature. This column gives the percentage of crossovers as calculated from the four classes of seeds.<sup>b</sup> Time is given in hours and minutes.

Thus in the progeny where the linkage is between *C* and *wx* the regression of percentage white on percentage waxy is  $-0.305$ . An increase of 6.14 in the percentage of waxy seeds should be accompanied, therefore, by a decrease of 1.87 in the percentage of white seeds. Actually the observed decrease was  $3.43 \pm 0.89$  per cent, not a bad agreement with expectation, and the error would permit of an actual difference of 0.76.

Assuming that the period of storage used on the plants shown in Table 17 had affected the gametes bearing the *c* gene to the same extent as was observed to be the case for the plants in Tables 20 and 21, then the difference in percentage white on ears resulting from the use of stored and fresh pollen should have been 0.03 per cent with the percentage of white in the stored pollen less than that of the fresh. This figure is well within the error of the observed difference and the agreement with the figure obtained by use of the regression coefficient may be entirely misleading.

#### POLLEN STORAGE AFFECTS THE CROSSOVER RATIO OF *C* AND *Wx*

Without exception, where due allowance is made for differences in time of storage and the observed increases corrected for the regression of difference on time, there is found to be a greater effect of storage on both *c* and *wx* when these factors are considered separately.

The data do not provide material for testing the effect of storage on the ratios of one pair of allelomorphs when the other pair is homozygous dominant, since in all instances where only one pair is heterozygous the other is homozygous recessive. This is a misfortune since it means that in those cases where the effect of storage on a single gene is measured it is in reality the effect of storage upon the double recessive combination *c wx*.

In all of the comparisons the effect of storage apparently increases both the *c* and *wx* genes to a greater extent when only one of the pair is heterozygous than when both are heterozygous, indicating that the selective action is more pronounced upon the double recessive combination than would be expected from the degree of selection found for the two genes separately.

This condition necessitates an alteration in the linkage between these two genes in the stored as compared with the fresh pollen. In the 13 ears reported by Collins and Kempton (5) it was concluded that storage failed to alter the crossover rate, or at least the effect was not greater than 2 per cent.

From the comparison of the effect of storage where *c* and *wx* are heterozygous with that found for these two factors separately, an alteration in the crossover rate approximating 2 per cent would be expected. Where the genes *c* and *wx* are linked any selective force that operated in such a manner as to increase the proportion of the gametes bearing the double recessive combinations would reduce the number of crossovers. Conversely, when *C* and *Wx* are linked a selective action that tended to increase both *c* and *wx* should increase the number of crossovers. An examination of Tables 16 and 17 shows this to be the case. As a result of pollen storage, crossing over is reduced in the group of ears where *c* and *wx* are known to be linked and is increased when *C* and *Wx* are linked. In neither instance is the difference large, but when the two differences are compared the

difference between them is  $3.73 \pm 0.95$ , a figure exceeding three times the error and probably significant.

This evidence, though slight, tends to confirm the conclusion that the combination of the genes *c* and *wx* is operated upon by some selective agent when the opportunity is presented by storing the pollen for several hours.

#### GAMETIC RATIOS UNALTERED IN POLLEN STORED OVER-NIGHT

Pollen from three heterozygous plants was collected in the afternoon, and approximately one-half of each lot was applied immediately to homozygous recessive plants. The remaining halves were stored over-night in the collection bags and applied the following morning to other homozygous recessive plants. The time of storage varied from 14 to 16 hours. Only a few such comparisons were made since it was believed to be very doubtful whether the pollen would remain viable, and the number of plants available for experimentation was limited.

The morning following storage the pollen was very unpromising in appearance, but nevertheless seeds were obtained on the three ears resulting from its use. The data are shown in Table 22.

In only two of the three cases was the percentage of waxy seeds obtained from the use of this stored pollen higher than that obtained with fresh pollen. The ears resulting from stored pollen are so small as to make the definite determination of any but very large differences impossible.

The data are presented here simply because it indicates that these long periods of storage at night do not bring about correspondingly large increases in the proportion of waxy to nonwaxy gametes.

TABLE 22.—Comparison of ears resulting from the use of fresh and stored pollen; pollen collected from the plants in the afternoon, one-half applied immediately to double recessive plants, the other stored overnight and applied the following morning

Plant No. (female)	Plant No. (male)	Pollen collected	Pollen applied	Number of seeds	Per cent white	Per cent waxy	Per cent crossing over
3642	3986	5.20 p. m.	5.20 p. m.	654	49.7 $\pm$ 1.32	48.3 $\pm$ 1.32	37.7 $\pm$ 1.28
3642-2	3986	5.20 p. m.	7.45 a. m.	191	45.0 $\pm$ 2.42	48.2 $\pm$ 2.43	35.6 $\pm$ 2.31
Difference		14 hours 25 minutes		463	-4.7 $\pm$	-1	-2.1
1593-2	1881	4.20 p. m.	4.20 p. m.	520	51.5 $\pm$ 1.96	48.5 $\pm$ 1.48	35.7 $\pm$ 1.41
1612	1881	4.20 p. m.	8.45 a. m.	61	50.0 $\pm$ 2.40	49.5 $\pm$ 2.40	38.2 $\pm$ 2.34
Difference		16 hours 25 minutes		459	-1.5	1.0	2.5
1624	1876	4.30 p. m.	4.30 p. m.	151	50.3 $\pm$ 2.74	45.0 $\pm$ 2.72	36.9 $\pm$ 2.64
1594	1876	4.30 p. m.	8.40 a. m.	56	41.1 $\pm$ 4.40	51.8 $\pm$ 4.50	38.5 $\pm$ 4.39
Difference		16 hours 10 minutes		95	-9.2	6.8	1.6

It should be borne in mind that this pollen was stored in the dark, thus eliminating any effect that light might have on stored pollen; though Brink's (3) experiments would seem to have eliminated light as a factor in the alteration of gametic ratios.

In considering these three cases it should also be borne in mind that the pollen was collected in the afternoon and the ratio of waxy to nonwaxy gametes may have receded from the high point even at the time of collection.

To this experiment may be added one other case where a tassel branch was removed from a plant and stored overnight with the lower end in water. The pollen was shaken out and applied the following morning. In the case of this plant the linkage was between *C* and *wx*. The ear obtained from this procedure may be compared with an ear resulting from the application of fresh pollen collected on the morning before the branch was removed, and also with an ear that resulted from the use of pollen collected in the morning and stored throughout that same day on the plant in the field, and, further, with a third ear resulting from the application of fresh pollen collected on the morning following the removal of the tassel branch.

The data from these ears are shown in Table 23.

TABLE 23.—Comparison of ears resulting from the use of fresh and stored pollen with an ear obtained by the use of pollen from a tassel branch kept for 15 hours and 45 minutes in water; the source of the pollen was a single plant heterozygous for *C* and *Wx* with the linkage between *C* and *wx*

POLLEN FROM ENTIRE TASSEL					
Pollen collected	Pollen applied	Number of seeds	Per cent white	Per cent waxy	Per cent crossing over
10.35 a. m., Aug. 15.....	10.35 a. m., Aug. 15.....	178	50.0±2.52	49.4±2.53	29.7±2.30
Do.....	4.35 p. m., Aug. 15.....	519	49.9±1.48	53.4±1.46	31.5±1.37
8.30 a. m., Aug. 16.....	8.30 a. m., Aug. 16.....	347	54.5±1.80	43.5±1.80	32.7±1.69
POLLEN FROM A TASSEL BRANCH					
4 p. m., Aug. 15.....	7.45 a. m., Aug. 16.....	285	41.4±1.96	50.2±2.00	30.8±1.84

The chief point of interest in this comparison is the fact that the percentage of waxy seeds on the ear resulting from pollen produced by a tassel branch stored overnight in water, is not increased above the expected 50 per cent.

TABLE 24.—Comparison of ears resulting from the use of fresh and stored pollen with an ear obtained by the use of pollen from a tassel branch kept for 23½ hours in 50 per cent alcohol; the source of the pollen was a single plant heterozygous for *C* and *Wx* with a linkage between *C* and *wx*

POLLEN FROM ENTIRE TASSEL					
Pollen collected	Pollen applied	Number of seeds	Per cent white	Per cent waxy	Per cent crossing over
8.30 a. m., Aug. 16.....	8.30 a. m., Aug. 16.....	347	54.5±1.80	43.5±1.80	32.7±1.69
9.55 a. m., Aug. 17.....	9.55 a. m., Aug. 17.....	293	50.2±1.97	43.7±1.95	33.0±1.86
Do.....	5.15 p. m., Aug. 17.....	229	36.7±2.15	68.6±2.07	28.5±2.01
POLLEN FROM A TASSEL BRANCH					
4.00 p. m., Aug. 16.....	3.30 p. m., Aug. 17.....	102	49.0±3.34	56.9±3.30	33.0±3.12

A very similar experiment was carried out with another tassel branch from this same plant, but instead of standing in water this tassel branch was put into 50 per cent alcohol and kept until 3.30

p. m. the following afternoon. The ear resulting from this treatment may be compared with an ear obtained with fresh pollen on the morning of the day the tassel branch was removed and with two other ears, one the result of fresh pollen applied in the morning after the branch was removed and the other the result of storing the pollen in the field on the day that the pollen from the removed branch was applied.

The ears are too small to permit any definite conclusions, but it is clear that the treatment accorded the tassel branch kept in alcohol did not affect the ratio of waxy to nonwaxy gametes beyond that found when stored pollen is used. The data are shown in Table 24.

### DISCUSSION

Although the fact that the storage of pollen increases the proportion of functioning gametes bearing the waxy gene and also the aleurone color gene *c*, may be considered as demonstrated, there is little evidence as to what conditions, present during storage, are responsible for the observed results.

Brink's (3) experiments with irradiation would seem to dispose of light as a factor in the alteration of the ratios, although in his experiments the proportion of waxy gametes increased with the increase in the period of irradiation. It is possible that the phenomenon is one of partial dehydration, though Brink partially desiccated the pollen in one experiment and obtained a slight decrease in the percentage of waxy. Attempts to gain an insight into the causal factors by cooling, drying, heating, etc., were made during the past season. In all but the cooling experiments these attempts resulted in complete failure to obtain seeds. This failure, however, can not be attributed entirely to treatment as the untreated controls also were largely failures, apparently owing to the protracted drought prevalent at the time pollinations were made.

Curiously enough, the only well-fertilized ears in the entire experiment, involving some 300 pollinations, were those that resulted from the application of pollen stored in desiccators at a temperature of 50° F. for a period of approximately seven hours.

As none of the applications of fresh untreated pollen produced ears of any consequence, no control is available with which to compare the cooled stored pollen. The ears produced as the result of fertilization with such pollen have abnormal waxy ratios entirely comparable with those obtained in the preceding years with pollen stored on the plant, but without controls it is not possible to determine whether the effect of cold storage is slightly greater or less than that of field storage.

Lacking more precise information as to actual causes it may not be amiss to make provisional deductions from the data.

In presenting the evidence for the effect of pollen storage on the waxy ratio, the difference between the ears resulting from fresh pollen as compared with those obtained with stored pollen has been stressed rather than the departure of the latter group from an expected percentage. This method of treatment was necessitated by the uncertainty as to the expected proportions, since in many progenies there is consistent deficiency of functioning waxy gametes in one sex or the other.

Placing the emphasis on the difference between the two sets of ears tends to obscure the fact that those ears resulting from fresh pollen have a deficiency of waxy seeds below equality almost as pronounced as the excess of waxy seeds on the ears resulting from stored pollen. This condition is shown in Figure 1.

The observed percentage of waxy seeds on all the ears resulting from the use of fresh pollen is  $46.79 \pm 0.30$ . The departure from the expected 50 per cent is 10.7 times the error. Pollen counts by Longley (9) and others have shown, even in very early stages, an equality of gametes bearing waxy to those having the dominant allelomorph. It is apparent that the proportion of gametes which fail to function is greater among those bearing the waxy gene than among those bearing the dominant allelomorph.

The deficiency of functioning gametes bearing the waxy gene in fresh pollen, considered in connection with the excess of such gametes in stored pollen, points to a slower rate of maturity for such gametes rather than to a higher death rate for gametes bearing the nonwaxy gene.

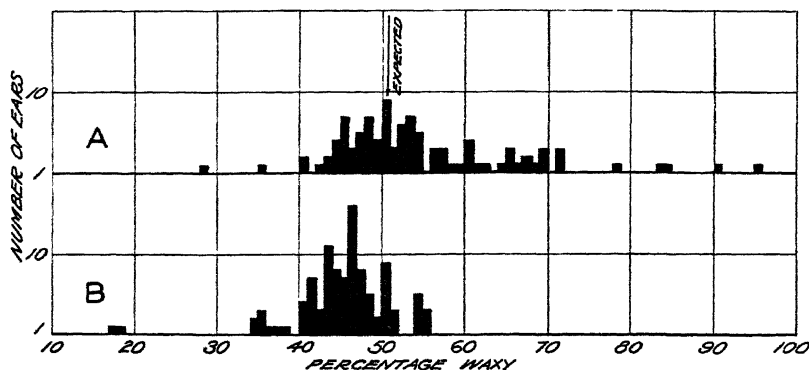


FIG. 1.—Frequency distributions of percentage of waxy seeds on ears obtained with fresh and stored pollen: A, stored pollen; B, fresh pollen

It is true that if the chemical nature of the gametes bearing waxy was such that these gametes absorbed or retained moisture, causing them to adhere to one another, a period of storage would provide time for them to become dry and to separate, thus increasing the effective gametes of this nature. Such a condition would result in restoring the ratio of waxy to nonwaxy from one of defect in fresh pollen to one of equality in stored pollen, but it would not result in increasing the ratio above equality unless accompanied by a loss of vitality of the gametes bearing the nonwaxy gene.

The simplest explanation then would seem to lie in the assumption that gametes bearing the *wx* and the *c* genes are not mature at the time of anthesis and that by the time they have attained maturity many of the gametes bearing the *Wx* or the *C* genes have lost their vitality.

Were the effect of storage restricted to the effect on the *wx* gene such an explanation might have merit since it is known that gametes bearing the *wx* gene differ chemically from those bearing *Wx*. But pollen storage seems also to have an effect on the *c* aleurone factor where a logical chemical explanation can only be surmised.

The gametes bearing the aleurone color gene *c* are not affected by storage to the same degree as those bearing the *Wx* gene, but the evidence seems conclusive that there is a tendency for such gametes to be in excess of equality in stored pollen. There is some evidence also that the functioning gametes bearing the *c* gene are less than equality in fresh pollen (fig. 2).

In calculating the mean percentage of white seeds on ears resulting from fresh pollen it has been necessary to eliminate ears that resulted from the application of pollen heterozygous for both *C* and *Wx*. In back crosses where the male parent is heterozygous for *C* and *Wx* and the linkage is between the dominant allelomorphs, a deficiency of waxy is accompanied by a deficiency of white and, conversely, if dominant and recessive genes are linked a deficiency of waxy results in an excess of colored. If such back crosses were not eliminated in

calculating the mean percentage of white seeds there would result only an indirect measure of the influence of the waxy gene upon the colored-white ratios.

For all ears resulting from fresh pollen not heterozygous for both *C* and *Wx*, the mean percentage of white seeds is found to be  $49.12 \pm 0.29$ , a departure from 50 per cent, just exceeding three times the error.

This result does not furnish conclusive proof that the ratio of gametes bearing *c* to those having the dominant allelomorph *C* is less than equality in fresh pollen.

It has been seen that pollen storage affects the gametes bearing

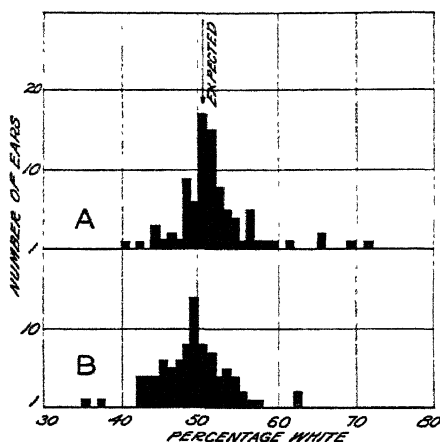


FIG. 2.—Frequency distributions of percentage of white seeds on ears obtained with fresh and stored pollen; A, stored pollen; B, fresh pollen

the *c* gene to a much less extent than it does gametes with the *wx* gene and apparently the *Cc* pair has less influence on the differential functioning of the gametes than the *Wxwx* pair.

With the number of ears available it is not possible to determine with reasonable certainty for the colored-white ratio departures, from the expected equality, of less than 1 per cent. Until further data are available, therefore, it would be unwise to assume that functioning gametes bearing the *c* gene are below equality in fresh pollen.

That the storage of pollen will be found to have an effect on the ratios of other genes seems a reasonable prediction justified by the results obtained with *wx* and *c*.

#### SUMMARY

When measured by differences in individual plant reciprocals there seems to be no basis for the conclusion that the ratio of waxy to nonwaxy bearing gametes is consistently less in the male than in the female gametes. Differences between the sexes with respect to

this pair of genes are found, but in some progenies the proportion of waxy is higher in the female and in others in the male gametes.

The percentage of waxy seeds is alike on upper and lower ears in back crosses where the male parent is heterozygous for the *Wx* gene. In back crosses where the female parent is heterozygous the percentage of waxy seeds is higher on the upper ears.

Apparently there is no difference in the percentage of waxy seeds on the butts and tips of the ears.

Long styles do not seem to be associated with a low percentage of waxy seeds.

Gametes bearing the genes *su* and *wx* seem to function as perfectly as those bearing other combinations of these genes.

The storage of pollen in the daylight for a period of approximately seven hours greatly increases the proportion of functioning gametes bearing the *wx* gene.

The storage of pollen for longer periods at night seems to have little effect on the proportion of *wx* to *Wx*.

Storage of pollen increases the proportion of gametes bearing the aleurone color gene *c*, but to a less degree than is found for *wx*.

The effect of pollen storage on the genes *c* and *wx* slightly alters the crossover ratio of these two genes. When the stored pollen is from plants with *c* and *wx* linked, the crossover percentage is reduced, and where the linkage is between *C* and *wx* the percentage of crossovers is increased.

Although the storage of pollen increases the proportion of *wx* to *Wx* and of *c* to *C*-bearing gametes to above equality, the proportion of these genes in fresh pollen is below equality.

From indirect evidence it is concluded that gametes bearing either the *wx* or *c* genes mature at a slower rate than those bearing the dominant allelomorphs.

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# SELECTION FOR QUALITY OF OIL IN SOY BEANS<sup>1</sup>

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## INTRODUCTION

In addition to its manifold uses in other fields of commercial activity soy-bean oil has of late years become an important factor in the paint industry. As a drying oil for paints it has passed beyond the experimental stage. In fact it is now possible to state that linseed oil, which is relatively expensive at present, can be replaced with an appreciable amount of soy-bean oil (20 to 25 per cent) to form a satisfactory oil for paints.

The drying quality of soy-bean oil is so much lower than that of linseed oil that the soy-bean oil can not be successfully used alone. It has therefore become highly desirable to attempt to increase the drying quality of the oil extracted from soy beans, for a higher quality of soy-bean oil means a smaller proportion of linseed oil for a proper drying mixture, and consequently a more economical oil for paints.

The most logical method of attempting to increase oil quality permanently in the soy bean (*Soja max*) is by breeding. This plant is well adapted for selection methods of breeding but less so for hybridization methods. This is true primarily because of the small size of the soy-bean flower, which renders crossing operations very difficult, but on the other hand, reduces the chances for contamination by cross-pollination of insects.

Experimental evidence shows that there is less than 0.2 per cent natural cross-pollination with the types under observation (18)<sup>4</sup>. Because the soy bean is so largely self-pollinated, the isolation of relatively pure strains or pure lines is often possible. It has repeatedly been demonstrated that soy beans can be grown successfully in the Northern States. In fact, they are especially well adapted to the sandy regions of Wisconsin. Since this is true, it is important to determine whether the cooler climate of Wisconsin is favorable to the production of high quality in soy beans. The work reported herein has an important bearing on this point.

## PLAN OF THE SELECTION EXPERIMENT

The experiment was begun in 1912 with a single soy-bean plant, the seeds of which were analyzed both for quality and percentage of oil. The progeny of this plant was then grown to maturity and the

<sup>1</sup> Received for publication Feb. 19, 1927; issued July 1, 1927. Paper No. 71 from the department of genetics, agricultural experiment station, University of Wisconsin. This contribution reports a portion of the results of a cooperative project between the department of genetics and the department of agricultural chemistry. The genetic work was inaugurated by L. J. Cole and O. Lloyd Jones in 1912 and, with the assistance of C. M. Woodworth, was carried on until the spring of 1919. At that time it was turned over to E. W. Lindstrom for continuation, and finally to completion and publication.

The chemical analyses were made under the direction of E. B. Hart, in charge of the agricultural chemistry, chiefly by E. M. Nelson, and also by J. S. Jurgens, G. D. Williams, D. W. Smith, and R. M. Bethke.

Financial assistance for a part of the chemical determinations was afforded by grants from the Paint Manufacturers' Association of the United States.

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<sup>4</sup> Reference is made by number (italic) to "Literature cited," p. 94.

seed from each individual was analyzed. From the highest and the lowest analyzing plants seeds were selected for planting the following year. These were the beginning of the high and low selection lines, respectively.

Their progeny in turn was analyzed. For each succeeding year the highest-analyzing plant of the high line was selected to continue that line. Likewise the lowest-analyzing plant of the low line was chosen for continuing the low line. This procedure was followed for each of seven years. Hence the entire progeny at the end of eight years could be traced back to the single original plant.

As a result of normal self-fertilization, this process would naturally tend to produce a pure line of beans, especially for the character being selected. At the same time other obvious characters, such as flower color, growth habit, maturity, and yield, would also tend to become uniform.

Since quality of oil is more important in paint manufacture than quantity, and since the number of chemical analyses was necessarily limited, it was decided to concentrate on one of the characters, namely, quality of oil. Consequently the high and low selections were made on the basis of the drying quality of oil. However, the quantity or percentage of oil produced by each plant was also determined in every case.

#### ANALYTICAL METHODS

Quality of soy-bean oil for paint manufacture depends upon its capacity for rapid drying. This means its ability to combine with atmospheric oxygen, and is dependent on the degree of unsaturation of the fatty acids that go to make up the oil. The best oil is that which is highly unsaturated, and hence combines quickly with oxygen to form a thin film over the paint.

Unsaturated oils absorbed elements other than oxygen. Iodine, for example, is very rapidly absorbed. Hence iodine can be used in determining the degree of unsaturation of oils, the amount of iodine absorbed being readily measured. The percentage of iodine absorbed by an oil is called its iodine number. This is found by various standardized methods such as those of Hübl, Hanus, or Wijs. Linseed oil has an average iodine number of 180, while commercial soy-bean oil averages less than 128. For the chemical analyses of oil quality in this experiment the method of Hübl was used.

For analytical purposes air-dried samples of 10 gm. taken at random from the harvested seed of individual plants were given to the chemist. Occasionally two or three samples from a single plant were included as a check on the chemical work. The samples were labeled with other than pedigree numbers, so as to prevent any unconscious personal equation from entering into the results. The iodine values quoted herein are believed to be accurate within the limits  $\pm 2$  and the determinations on percentage of oil to  $\pm 0.3$  per cent.

#### FIELD METHODS

The experimental soy beans were grown in a clay-loam soil in the genetics plots at the University of Wisconsin. Except in 1913, the soil of each new plot was inoculated with soy-bean bacteria. The plots were manured lightly and plowed yearly.

Planting was all done by hand in the latter part of May or earlier. The rows were 3 feet apart, and the individual plants were spaced exactly 2 feet apart in the row. Each row was properly staked, and when the plants were mature each was labeled with a tag bearing its pedigree number.

Usually 50 or more seeds from each selected plant were grown to maturity. As far as possible these were grown in duplicate rows planted in different parts of the plot. Notes on flower color, growth habit, pubescence, and maturity were taken for each plant. These various characters served as excellent checks on the relative purity of the strains.

Harvesting the seed was done entirely by hand, the plants being pulled or cut separately. After harvesting they were taken to the seed house and dried for a period of three weeks or longer.

The plants were then threshed by careful workers. Each plant with its tag was run through a small threshing machine especially devised for this work. The construction was such that it was practically impossible for a bean to lodge in any of the various parts and so contaminate the plant that followed.

It is firmly believed that errors of mechanical mixing of seed and erroneous labeling have been reduced to a negligible minimum, since the person directly in charge of the experiment was in close personal touch with the work at all stages. Careful note-taking on the many morphological characters has made it possible to detect any very evident mixtures in the field, since the two selection lines differed in some of these characters.

#### CHEMICAL COMPOSITION OF THE SOY BEAN

While the interest of this report centers chiefly in the relation of soy-bean oil to paint manufacture, it seems advisable to indicate briefly the general chemical composition of the soy bean. A brief comparison between its composition and that of other oil-producing seeds has accordingly been arranged (Table 1).

TABLE 1.—*Chemical composition of various oil-producing seeds*

Seed	Source of data	Oil	Water	Ash	Crude protein	Fiber	N-free extract
		<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Soy bean.....	Henry and Morrison (?).....	17.5	9.9	5.3	36.5	4.3	26.5
Do.....	Delaware Bul. 99 (14).....	17.2	11.7	4.8	33.5	4.5	28.3
Do.....	U. S. Dept. Agr. Farmers' Bul. 58 (9, 16).....	16.9	10.8	5.8	34.0	4.6	28.7
Flaxseed.....	Henry and Morrison (?).....	33.7	9.2	4.3	22.6	7.1	23.2
Do.....	North Dakota Bul. 118 (15).....	37.5	5.0		24.6		
Do.....	do.....	39.3	6.7				
Cottonseed.....	Henry and Morrison (?).....	19.0	9.4	4.6	19.5	22.6	24.9
Do.....	Delaware Bul. 99 (14).....	19.9	10.3	3.5	18.4	23.2	24.7
Dent corn.....	Henry and Morrison (?).....	5.0	10.5	1.5	10.1	2.0	70.9
Sweet corn.....	do.....	7.9	9.3	1.8	11.5	2.3	67.2
Sunflower.....	do.....	24.7	6.9	3.1	16.1	27.9	21.3

The soy bean averages 17 to 18 per cent oil, which is considerably lower than flaxseed and much higher than corn. Analyses reported by Fellers (3) for a large number of soy bean varieties are given in Table 2. These analyses show that there is a distinct varietal dif-

ference not only in percentage of oil but also in percentage of protein. Some well-known varieties like Auburn, Manchú, Guelph, Black Eyebrow, and Medium Yellow yield high in oil as well as in protein. This is an important consideration where soy beans are used for feed as well as for oil. Incidentally, Fellers believes that the oil is more easily pressed from some varieties than from others.

TABLE 2.—Average percentage of oil and protein in different varieties of soy beans; from Fellers (3)

Variety	Number of samples	Average per cent of oil	Average per cent of protein	Variety	Number of samples	Average per cent of oil	Average per cent of protein
Manchuria.....	3	23.2	36.9	Haberlandt.....	6	19.2	38.3
Auburn.....	3	21.6	40.0	Ito San.....	6	19.0	41.4
Manchu.....	6	21.6	40.5	Wilson.....	6	18.8	37.3
Ohio 1935.....	6	21.5	37.3	Manhattan.....	4	18.5	37.9
Guelph.....	8	21.2	38.1	Peking.....	6	17.9	37.3
Black Eyebrow.....	172	20.2	38.7	Hollybrook.....	6	17.2	40.8
Medium Yellow.....	6	19.6	39.3	Arlington.....	6	16.7	43.4
Early Brown.....	16	19.5	40.3	Ebony.....	8	14.6	43.6

It has been reported that an average bushel of soy beans contains about 11 pounds, or 1.42 gallons, of oil. At present oil mills are expressing only 70 to 75 per cent of the total amount of oil contained in the beans. By the proper use of a solvent, such as benzol, practically all of the oil might be removed.

The oil of the soy bean normally is practically neutral, and about 95 per cent is saponifiable. It is very similar to linseed oil. The oil itself consists of about 95 per cent of glycerol esters of the fatty acids. The oleic, linoleic, and linolenic acids are the unsaturated acids that are responsible for the drying quality of the oil. Their various proportions are given in terms of percentage as follows:

Oleic acid.....	{26.5 (Smith).
	{33.4 (Baughman and Jamieson).
Linoleic acid.....	{56.6 (Smith).
	{51.5 (Baughman and Jamieson).
Linolenic acid.....	{2.4 (Smith).
	{2.3 (Baughman and Jamieson).

The oil of the soy bean is classed as a semidrying oil, being more efficient than cottonseed oil and less so than linseed oil. After treatment with certain driers, it is satisfactorily mixed with linseed oil if the proportion of soy-bean oil does not exceed 20 to 25 per cent.

Published data on the iodine values of soy beans, indicating the drying quality of the oil as compared with other oils, are recorded in Table 3.

The average iodine number of soy-bean oil can probably be given as 128 to 130, although ordinary commercial oil often tests somewhat lower. Linseed oil is markedly higher, and cottonseed and corn oil are much lower. There is considerable variability among the different varieties of soy beans in their iodine value as well as among different samples of the same variety. In general, it is true that there is no significant difference between imported and domestic beans.

TABLE 3.—Iodine numbers of oil of different seeds

Oil	Source of data	Number of samples analyzed	Iodine number
Soy bean:			
Domestic.....	Delaware Bul. 99 (14).....	48	129.7
Do.....	Paint Manfrs. Assoc. Circ. 37 (10).....	5	125.8
Imported.....	do.....	2	128.8
Do.....	Paint Manfrs. Assoc. Circ. 67 (4).....	7	130.7
Do.....	do.....	9	134.5
Ito San.....	North Dakota Bul. 118 (15).....	17	131.2
Haberlandt.....	do.....	8	129.2
Wilson.....	do.....	17	129.0
Manchuria.....	do.....	31	126.6
Mammoth Yellow.....	Baughman and Jameson (1).....		128.0
Linseed.....	Delaware Bul. 99 (14).....		180.0
Do.....	Paint Manfrs. Assoc. Circ. 67 (4).....	3	189.3
Do.....	North Dakota Bul. 118 (15).....	48	185.9
Cottonseed.....	Delaware Bul. 99 (14).....		108.0
Corn.....	do.....		119.2

## PREVIOUS WORK ON THE HEREDITY OF CHEMICAL CHARACTERS

Except for experiments on plant pigments and considerable practical breeding for sugar in beets, there has been little direct research on the inheritance of chemical characters in plants.

The Illinois corn-breeding experiment (12) on selection for high and low protein and high and low oil content stands preeminent in this field. By means of simple mass selection, four strains of corn were isolated after 10 years of breeding, a high-oil and a low-oil, a high-protein and a low-protein strain.

By analyzing the pedigree records of the Illinois experiment, Surface (13) was able to show that the selection process had merely isolated already existing types of oil and protein percentage, the intermediate types having been discarded during the years of selection. This process is characteristic of an open-pollinated crop like corn.

Pearl and Bartlett (11) describe a preliminary undertaking on the heredity of chemical characters in maize. From their experiments they infer that such seed characters as moisture, nitrogen, protein, crude fat, ash, crude fiber, pentosans, sucrose, dextrose, and starch are inherited in maize essentially in accordance with Mendelian principles. They state that "probably each of the characters, protein, crude fat, and ash content, segregates as a definite and distinct unit character." However, their evidence is limited and their conclusions remain unchecked. All present evidence indicates a far more complex situation. Especially is this true of protein, as the work of East and Jones (2) proves.

Woodhouse and Taylor (17) report some interesting and critical results obtained in experiments on the relation between nitrogen content and oil content of soy beans. In these experiments they were apparently working with pure varieties, and their analyses are based on seed from single plants.

The varieties or types of soy beans used were named according to seed-coat characteristics, black, yellow, and chocolate. The vegetative characters of the yellow and chocolate types were similar, but

distinctly different from those of the black type. A summary of the findings that bear on the present problem follows:

Variety	Nitrogen, per cent	Oil, per cent	Correlation, nitrogen and oil
Black.....	6.72±0.02	13.52±0.08	-0.25±0.07
Yellow.....	5.61±.02	16.99±.07	-.34±.07
Chocolate.....	5.87±.02	17.13±.09	-.33±.07

It is evident that the black variety is distinctly different from either of the other two, both in nitrogen and in oil content. This difference is statistically significant, as the writers have determined by calculating the probable errors of the differences. For example, between the black and yellow varieties there is a difference in nitrogen of 1.11 per cent, the probable error of which is 0.03 per cent. For oil content the difference between these varieties is 3.47 per cent, with a probable error of 1.05 per cent.

It is interesting to note that a slight negative correlation exists between nitrogen and oil content in all three varieties. In other words, a high oil content apparently means a slightly lower nitrogen content. Woodhouse and Taylor stress the point that the correlation is not very large. They are inclined to believe that "the percentage content of nitrogen and oil is in all probability a characteristic that is inherited." They also note that selection for extreme percentages of nitrogen and oil did not affect the chemical content of the progeny in these respects in the following year.

Fellers (3) is inclined to believe that, in general, varieties with a high percentage of protein (or nitrogen) show a slightly lower percentage of oil. By calculating the coefficient of correlation for protein and oil from his data for 25 varieties, a fairly high negative correlation is found ( $-0.56 \pm 0.09$ ). But in view of the nature of the material, which was a heterogeneous mixture of varieties, the value of such a correlation is extremely uncertain.

Similarly, from certain data given by Grantham (6) the writers have calculated the correlation between percentage of protein and oil. These data are given in the form of chemical analyses for 51 varieties of beans, a single determination for each variety. To determine a correlation in such a mixture of types is often hazardous, but in view of Fellers (3) and Woodhouse and Taylor's (17) results in a similar situation, these data seemed to afford an interesting check. The correlation between these two characters was found to be  $-0.02 \pm 0.09$ . This negative correlation is so small, however, especially in comparison with its probable error, that no evident relation can be said to exist between percentage of protein and percentage of oil.

Ladd (8), in chemical analyses for oil quality and percentage of oil in the soy bean, finds that the iodine number of a certain soy-bean variety is somewhat changed by the locality in which the plants are grown. Limited data indicate that "when beans giving a certain iodine value in one locality are then grown in a colder climate they show a decided increase in the iodine number. When soy beans from the North are grown in a warmer climate the iodine value drops." He also states that "an increase or decrease in iodine value

seemed to have no consistent effect on the oil content nor does the oil content seem to affect the iodine number." These conclusions have an important bearing on the results of the investigations reported herein.

It is worthy of note that both the results of Garner, Allard, and Foubert (5) and of Fellers (3) show that early and late plantings of the same variety produce some differences in the percentage of oil. The very late plantings showed a decrease in oil percentage. The first-named investigators are not inclined to regard the difference as due primarily to the date of planting, however, but rather to the character of the season itself. They found no correlation between size of seed and percentage of oil. The addition of phosphorus to pot cultures seemed to increase the oil content, but potassium had no effect. Under practical conditions, they were inclined to believe that climate is a more important factor than soil type in affecting the oil content of soy beans.

Fellers (3) remarks that in his experiments phosphates increased the protein content of soy-bean seeds at the expense of oil and that lime (oxide or carbonate) depressed the oil content. He also maintains that the inoculation of soy-bean plants with *Bacillus radicicola* caused a marked decrease in oil content.

#### EXPERIMENTAL RESULTS OF SELECTION FOR OIL QUALITY

As a preliminary step in the selection experiments, 11 varieties or strains of soy beans from various sources were analyzed in 1912 for their iodine number and percentage of oil. The results of these preliminary tests are recorded in Table 4.

TABLE 4.—Chemical analyses of oil in soy beans of different varieties in 1912

Variety or B. P. I. No. <sup>a</sup>	Number of plants analyzed	Average per cent oil	Average iodine number	Range in per cent oil	Range in iodine number
Wisconsin.....	5	18.0	127.6	17.4 to 18.8	122.2 to 132.5
Brown.....					
17257.....	5	16.8	133.1	12.6 to 19.2	127.4 to 136.3
17258.....	6	19.3	128.8	17.2 to 23.6	124.2 to 133.7
17288c.....	4	18.1	131.2	17.1 to 18.9	122.2 to 137.7
17277.....	5	19.0	123.6	17.2 to 21.0	109.9 to 132.4
18227.....	6	17.3	130.1	14.0 to 20.0	125.3 to 137.8
20405.....	5	17.3	129.6	13.9 to 19.1	121.0 to 139.6
20406.....	5	19.9	130.1	18.4 to 21.3	126.0 to 133.2
20551.....	7	20.1	122.4	17.7 to 22.1	117.0 to 128.0
Illinois.....	2	17.8	128.2	16.5 to 19.2	127.6 to 128.9
Yellow.....					
Wisconsin.....	4	19.1	126.0	18.0 to 20.2	122.2 to 128.0
Brown.....					

<sup>a</sup> Bureau of Plant Industry, U. S. Department of Agriculture.

As a result of these analyses, B. P. I. No. 17277, which showed the greatest range of variability in iodine number, was chosen as a starting point for the selection experiments. In view of the complex variability of this variety in the succeeding years, it would perhaps have been wiser to choose a variety that was purer for its morphological characters.

The seed of No. 17277 was obtained from the department of agronomy at the University of Wisconsin, having come originally

from the Bureau of Plant Industry of the United States Department of Agriculture. The variety name at that time was Manhattan. The seeds were yellow, with traces of brownish seed-coat pigment around the hilum. This variety was far from pure, for there was variation in flower color (purple and white), in pubescence color (tawny and gray), in growth habit (tall and dwarf), and in time of maturity.

Five plants of No. 17277 were analyzed in 1912. Of these, one plant, Wisconsin pedigree No. 1225c, which analyzed highest in iodine number (132.4) was chosen to continue the selection experiment.

The seed of 1225c was grown in 1913. Again variability of the strain appeared, especially in flower color and growth habit. Chemical analyses of 20 plants taken at random among the progeny were made, and the results recorded (Table 5).

TABLE 5.—Iodine number and percentage of oil of progeny of original plant 1225c, 1913

Plant No	Iodine number	Per cent of oil	Plant No.	Iodine number	Per cent of oil
1325c-100.....	133.0	17.7	1325c-88.....	126.7	17.5
1325c-142.....	131.5	18.1	1325c-148.....	128.7	17.2
1325c-136.....	130.5	18.4	1325c-90.....	126.3	17.8
1325c-110.....	130.5	19.2	1325c-80.....	125.6	17.7
1325c-64.....	130.2	19.5	1325c-88.....	124.6	17.2
1325c-112.....	129.5	17.0	1325c-84.....	124.5	20.0
1325c-122.....	129.5	17.9	1325c-62.....	124.3	17.6
1325c-144.....	129.5	18.4	1325c-152.....	117.1	18.9
1325c-138.....	125.0	18.7	1325c-132.....	107.5	14.2
1325c-164.....	127.6	19.3			
1325c-60.....	127.1	18.7	Average.....	126.5	18.0

Although considerable variability in iodine number was manifest, only one plant surpassed the analysis of the mother plant. This plant, 1325c-100, with an iodine number of 133, was consequently chosen as the starting point of the high line. The plant lowest in iodine number, 1325c-132 (107.5), was chosen to serve as the beginning of the low line.

From this point selections for high and low iodine number were made in each of the following years: 1914, 1915, 1916, 1917, 1918, 1919, and 1920. In each year the plant of the high line having the highest iodine value was selected to continue the high line, and the plant of the low line having the lowest iodine value was used to continue the low line. Thus selections were made on iodine value and not on percentage of oil, although the latter was always determined.

The average (mean) yearly iodine number with its probable error, and the variability of each year's progeny as measured by the standard deviation, are listed in Table 6.

TABLE 6.—Means and standard deviations of eight years' data for high and low oil selection in soy beans in terms of iodine number

Pedigree No.	Year grown	Number of plants analyzed	Selection line	Mean (average) iodine number	Standard deviation
1325e-100.....	1913	1	High.....	133.0	-----
1325e-132.....	1913	1	Low.....	107.5	-----
5.....	1914	20	High.....	137.1 $\pm$ 0.5	3.1 $\pm$ 0.3
6.....	1914	20	Low.....	135.9 $\pm$ .3	2.3 $\pm$ .2
99.....	1915	14	High.....	125.4 $\pm$ 1.2	6.5 $\pm$ .8
98.....	1915	8	Low.....	131.4 $\pm$ .6	2.3 $\pm$ .4
207.....	1916	50	High.....	130.2 $\pm$ .4	3.8 $\pm$ .3
212-3.....	1916	20	Low.....	128.4 $\pm$ .4	2.4 $\pm$ .3
400.....	1917	87	High.....	134.5 $\pm$ .2	2.4 $\pm$ .1
401.....	1917	44	Low.....	132.2 $\pm$ .2	2.3 $\pm$ .2
866.....	1918	61	High.....	136.6 $\pm$ .4	4.6 $\pm$ .3
867.....	1918	45	Low.....	124.9 $\pm$ .2	2.2 $\pm$ .2
1129.....	1919	34	High.....	131.9 $\pm$ .5	4.2 $\pm$ .3
1131-2.....	1919	41	Low.....	124.5 $\pm$ .3	3.3 $\pm$ .2
1325.....	1920	63	High.....	132.5 $\pm$ .3	3.5 $\pm$ .2
1331.....	1920	16	Low.....	125.2 $\pm$ .8	4.5 $\pm$ .5

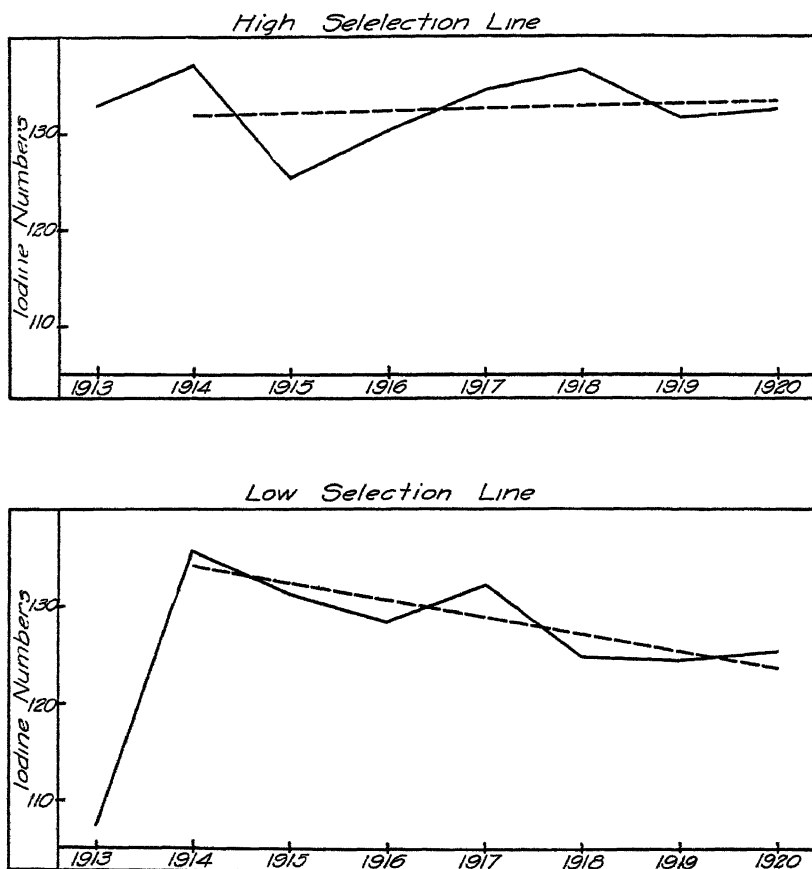


FIG. 1.—Yearly averages of iodine numbers in the high and the low selection lines of soy beans for a period of seven years (1913-1920). The broken line is a fitted straight line to indicate general trend

The general trend of the eight years' data on selection for iodine number is most easily visualized by plotting graphically the yearly averages, as has been done in Figure 1. In this graph the high and low lines have been plotted separately, and a straight line has been fitted to the data by the method of least squares. This line should indicate the general trend of the results, which is somewhat obscured by seasonal or yearly fluctuations such as are common with all determinations of chemical characters in plants. In fact, as will be shown later, these fluctuations are a serious obstacle to any selection work, since they often obliterate the true genetic nature of the plant.

The general trend of the data on iodine values for the high line during the seven years of selection seen in Figure 1 shows a slight upward tendency. It indicates that some slight progress has been made in increasing the iodine number of the variety. But the increase is so small that its real significance might be somewhat in question. When compared with the original average of 123.6 (Table 4), however, there is perhaps no doubt that the increase is significant. From a commercial standpoint such an increase is of doubtful value, since it has not surpassed the already existing quality of soy-bean oil to an appreciable extent.

A glance at the lower half of Figure 1 reveals a distinct downward tendency of the low line. If all the data are considered (including the abnormally low value of 107.5 of the single plant from which the line originated) a fitted line shows a slight upward trend. This obviously does not fit the facts of the case, for this upward tendency is entirely occasioned by the first ordinate in the graph. This point, which is that of only a single-plant analysis (and that most certainly an abnormal one, as shown by the distinct jump in the following year 1914), should not be permitted to influence the rest of the data to such an extent as it does. Accordingly, a straight line has been fitted to the data of the years 1914-1920, each ordinate of which is represented by an average of a fair sample of analyzed plants and not of a single plant. When this is done a pronounced downward trend is noted. This undoubtedly approximates the true condition.

In order to provide a more detailed analysis of the data, the differences between the averages (means) of the high and low lines for the successive years of selection have been arranged (Table 7).

TABLE 7.—*Difference between the average iodine numbers of the high and low selection lines*

Year	Selection line	Mean iodine number	Difference <sup>a</sup>	Year	Selection line	Mean iodine number	Difference <sup>a</sup>
1913	(High.....)	133.0	25.5	1917	(High.....)	134.5±0.2	2.3±0.3
	(Low.....)	107.5			(Low.....)	132.2±.2	
1914	(High.....)	137.1±0.5	1.2±0.6	1918	(High.....)	136.6±.4	11.7±.4
	(Low.....)	135.9±.3			(Low.....)	124.9±.2	
1915	(High.....)	125.4±1.2	-6.0±1.3	1919	(High.....)	131.9±.5	7.4±.6
	(Low.....)	131.4±.6			(Low.....)	124.5±.3	
1916	(High.....)	130.2±.4	1.8±.6	1920	(High.....)	132.5±.3	7.3±.9
	(Low.....)	128.4±.4			(Low.....)	125.2±.8	

<sup>a</sup> Any correlation existing between the two variables has been disregarded in calculating the probable error of the difference.

The last column in this table shows that during the early years of the experiment (1914-1916, inclusive) there was no constant or significant difference in iodine value between the high and low lines. Commencing in 1917, however, the difference attains a statistical significance in comparison with its probable error, and this is continued in the following years. In the last two years (1919 and 1920) the difference appears to have become constant. Certainly it is mathematically significant; and there is probably not much doubt that the high and low lines have been noticeably separated during the course of selection.

From the experimental data presented above one might deduce that the selection methods had actually changed the chemical constitution of the oil in the original variety of soy bean. But another phase of the experiment seems to reveal the underlying cause.

This new phase of the problem is concerned with a certain morphological or physiological character of the soy-bean plant and its apparent relation to the iodine value of the oil. It has been stated that the original variety from which the high and low selection lines were developed was, unfortunately, not pure for certain botanical characteristics, notably flower color, pubescence, maturity, and growth habit. Further, it has been noted that the selection process eventually rendered the two lines pure for certain botanical characters.

At the present time the high line is characterized by purple flowers, gray pubescence, lateness of maturity, and a tall, somewhat indeterminate habit of growth. The line breeds true for these characters. The low line, on the other hand, is pure for white flowers, gray pubescence, and a dwarf, stocky, determinate habit of growth. The low line is also fully two weeks earlier than the high. The distinction in growth habit is clearly seen in Figure 2, which shows a high and a low line row of plants growing side by side in 1922.

By 1916 the original mixture of botanical characters was straightened out as far as the low line was concerned. As to flower color, this low line proved to be white from the very beginning. The growth habit, which at that time was more difficult of determination because of variation, was not certainly pure until 1916, when it was noted as 100 per cent dwarf; and this was accompanied by an early maturity.

The high line, on the other hand, continued to throw the dwarf type in a definite proportion (approximately 25 per cent, being a simple Mendelian recessive) until and including the year 1919. In 1920, however, it was decided to render it pure for this growth habit. Accordingly, 10 of the highest-analyzing plants of 1919 were selected and grown in progeny rows the following year. Four of these proved to be 100 per cent tall. They repeated this process uniformly in the following seasons of 1921 and 1922.

As the matter now stands, these selections for plant characteristics, by removing the secondary modifiers of growth habit, have produced two unusually distinct and uniform lines of plants. The high line is remarkably uniform for growth habit (tall and somewhat indeterminate), purple flower color, and time of maturity, being distinctly late or medium late. During the season of 1922 evidences of mosaic rendered an otherwise vigorous strain somewhat unhealthy in appearance, but apparently did not influence the yield to any extent. This high line would be classed as a more than average yielder of seed.

The low line is characterized by a stocky, bushy habit of growth, white flowers, and very early maturity. It is a very poor yielder in

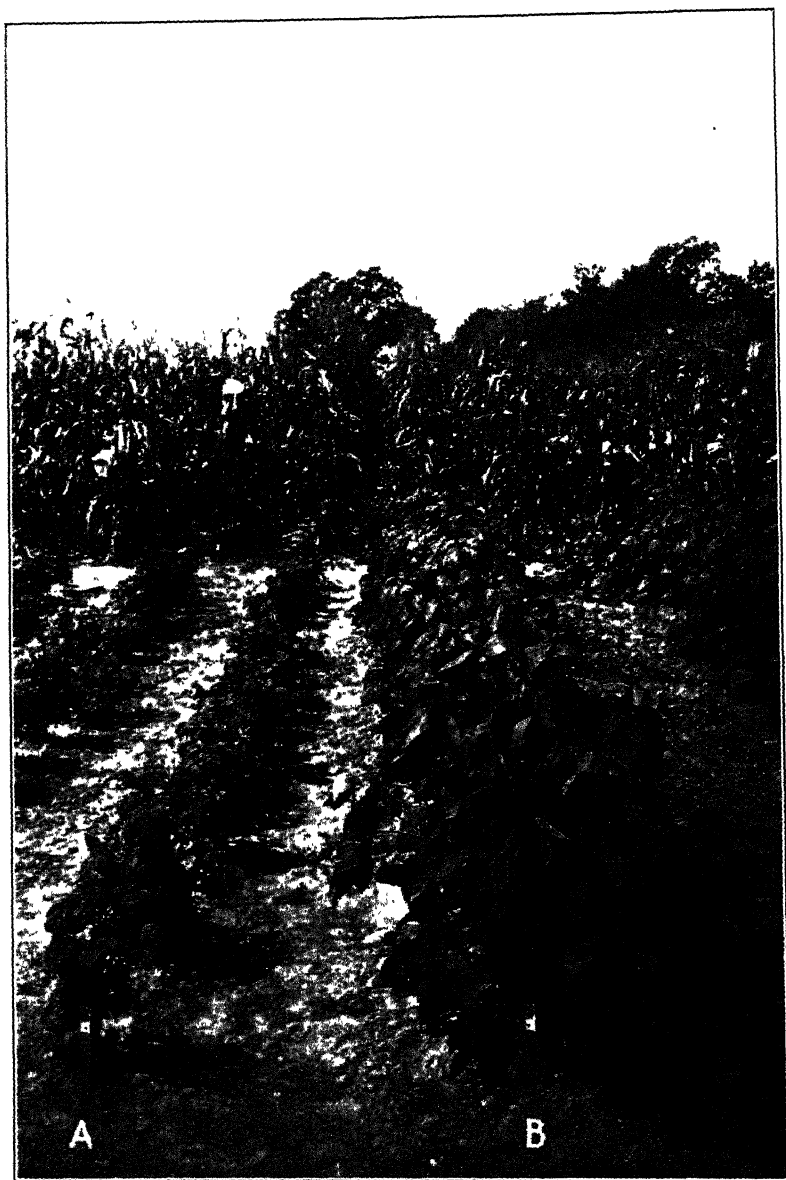


FIG. 2—A, row of low-line selection of soy bean, showing early dwarf type; B, row of high line, showing the late tall type

most seasons. The low line beans show much greater development of a brownish pigment of the seed coat around the hilum than do those of the high line.

The point at issue, however, is the relation between these botanical characteristics and oil production. It has been noted that apparently the difference produced by high and low selection for iodine number (an average difference of about seven to eight in the last three years) was caused by a slight increase in the high line and a marked decrease in the low line. With this in mind a survey of the data should reveal something of interest.

A glance at Table 7 shows that in the years 1914 to 1917 there was no great difference in average iodine number between the high and low lines, except in 1915, when an actual difference in favor of the low line occurred (for which there is no accountable reason). There was an average difference of about two points in favor of the high line for the years 1916 and 1917. Later, in 1918-1920, this difference was markedly accentuated.

However, when the records, both botanical and chemical, are scrutinized carefully a possible explanation for this phenomenon appears. If the analyses within the high line are separated so as to group the late tall type of plant separately from the early dwarf type occurring in that line during those years, it is apparent that there are actually two groups chemically; the one with the higher analyses coming from the tall late plants and the other, which is considerably lower, from the early dwarf plants.

This fact is conclusively shown in Table 8, in which the iodine numbers of the late tall plants are contrasted with those of the early dwarf plants. The data are taken from the 1916, 1917, 1918, 1919, and 1920 crops, since before those years the distinction between the two plant types in the high line was not accurately enough determined to permit certain classification.

TABLE 8.—*Iodine numbers of late tall and early dwarf types of soy beans*

Year grown	Late tall type (all from high line)		Early dwarf type (from high and low line)		Difference
	Number of plants	Average iodine number	Number of plants	Average iodine number	
1916.....	44	130.5	26	128.9	1.6
1917.....	66	136.5	64	131.9	4.6
1918.....	51	137.9	55	126.2	11.7
1919.....	50	133.4	66	124.4	9.0
1920.....	63	132.5	15	125.2	7.3

It will be noted that, beginning in 1916, the late tall plants show a consistently higher iodine number than the early dwarf plants. This difference becomes marked in 1918, and from that point continues to be statistically significant. It so happens that the 1920 data show the least difference. The excess shown by the high line ( $132.5 \pm 0.3$ )—which in that year consisted only of the late tall type—over the low line ( $125.2 \pm 0.2$ ), consisting only of the early dwarf type, is  $7.3 \pm 0.9$ . The difference is eight times its probable error, and is of real statistical significance.

From this we are forced to conclude that the separation of the high and low lines in the selection process is in all likelihood due pri-

marily to the isolation of a low-analyzing strain characterized by an early dwarf type of plant. Whether the correlation existing is between quality of oil and earliness (and lateness), or tallness and dwarfness is uncertain. The following indirect evidence seems, however, to throw some light on the problem.

Two sets of 10 plants each of pedigree 207 were chosen for analysis at different stages of maturity. About 10 pods were picked from each plant. The first picking was made on August 23, when the seeds and pods were green; the second on September 8, when the pods were turning yellow; and the third on September 18, after the plants were fully mature and the seeds thoroughly dry.

TABLE 9.—*Chemical analyses of soy beans at different stages of maturity*

Set No.	Date of picking	Average per cent of oil	Iodine number	Per cent of water, dry basis	Stage of ripeness
1.....	Aug. 23	19.06	124.9	71.6	Pods and seeds green.
1.....	Sept. 8	24.11	125.6	41.0	Pods turning yellow.
1.....	Sept. 18	18.41	128.8	-----	Seeds ripe and dry.
2.....	Aug. 23	19.30	126.5	71.1	Pods and seeds green.
2.....	Sept. 8	24.17	124.6	40.3	Pods turning yellow.
2.....	Sept. 18	18.17	129.1	-----	Seeds ripe and dry.

The analyses of the seed from these two sets of plants are shown in Table 9. The two sets check each other fairly well. It is significant that the iodine number increases quickly at the last stage of maturity, there being very little difference in the early stages.

One explanation which may be offered to account for the fact that the late, high line has a higher iodine value than the early low line is that the quick maturity of the low line may possibly retard the complete development of the unsaturated oils, whereas the slower maturity of the high line may provide better conditions for the oil formation.

A further explanation is offered in view of the experience of Ladd (8). He found that a colder climate markedly increased the iodine number of a variety, and that when northern varieties of soy beans were grown in the South, a noticeable decrease in the iodine value occurred. Accordingly, the cooler weather prevailing at the time the high line matured might probably have influenced the iodine value, since the lower temperatures occurred at a time when, as the experiments indicated, a rapid change in iodine value was taking place. The climate of Wisconsin becomes noticeably cooler during September, especially at night; so that a difference in maturity of two weeks at this time might perhaps be critical in the development of the unsaturated oils. For this reason it seems logical to assume that the correlation noted above exists between high iodine value and late maturity (or low iodine value and early maturity). If this assumption is true, the soy beans of highest quality must necessarily be the later-maturing sort, or they must be grown so that their critical stage of maturity coincides with cool weather. This is a matter that can readily be tested by experiment.<sup>4</sup>

<sup>4</sup> This point was subjected to a test in 1923, but because of adverse circumstances in the field, the value of the material for experimental purposes was destroyed. Attempts were made to verify the hypothesis later, but conditions prevented it. After much delay this report is presented without verification of this point.

## VARIABILITY IN IODINE VALUES DURING THE PROCESS OF SELECTION

Ordinarily the continued selection for any one character tends to produce a more uniform progeny in respect to that character, and this is especially true of pedigree selection such as was practiced in this experiment. It is a noticeable fact, however, that the variability of the iodine numbers did not decrease materially as the selection process went on (Table 6). Perhaps the obvious reason for this inconsistency is the fact that a chemical character like quality of oil is extremely susceptible to external conditions. Climatic conditions for example, are known to exert a very marked effect upon it. Hence some of the variability shown in the early stages of the experiment might be attributed to such causes, and not all to the heterozygous genetic condition of the soy bean. Selection would then cause no appreciable change in the variability, especially after the plant type was fixed.

For this reason successful selection for high (or low) quality of oil is very difficult. The true genetic potentiality of the plant is so obscured by these temporary fluctuations caused by the immediate environment that the selection for high or low quality is rarely based on heritable variations that would make for progress.

TABLE 10.—Means and standard deviations of eight years' data on percentage of oil in soy beans

Pedigree No.	Year grown	Number of plants analyzed	Selection line	Mean (average) per cent of oil	Standard deviation
1325c-100	1913	1	High	17.7	
1325c-132	1913	1	Low	14.2	
5	1914	20	High	14.8±0.1	0.8±0.1
6	1914	20	Low	15.5±.1	.8±.1
99	1915	14	High	15.1±.2	1.0±.1
98	1915	8	Low	14.3±.2	.8±.1
207	1916	50	High	16.2±.1	1.4±.1
212-3	1916	20	Low	17.5±.1	.6±.1
400	1917	87	High	14.0±.1	1.5±.1
401	1917	44	Low	13.7±.1	.7±.1
866	1918	61	High	16.8±.1	1.1±.1
867	1918	45	Low	16.2±.1	1.4±.1
1129	1919	34	High	17.1±.1	1.1±.1
1131-2	1919	40	Low	15.4±.1	1.3±.1
1325	1920	64	High	16.7±.1	.7±.1
1331	1920	16	Low	14.6±.2	1.3±.1

A glance at Table 10 shows that the variability in oil percentage was also unchanged during the course of the experiment. This is not strange since there was no attempt to select on the basis of oil percentage.

It should be noted that the many morphological characters of the soy-bean plants were gradually rendered less variable during the eight years' time. Apparently external or climatic factors influence such characters much less readily than they do the chemical characters.

## RELATION BETWEEN QUANTITY AND QUALITY OF OIL IN THE SELECTION EXPERIMENTS

As has been stated, while the selection for high and low quality of oil was in progress, the amount or percentage of oil per plant was likewise determined. The figures thus obtained offer an excellent criterion by which to detect any relation between quantity and quality of oil.

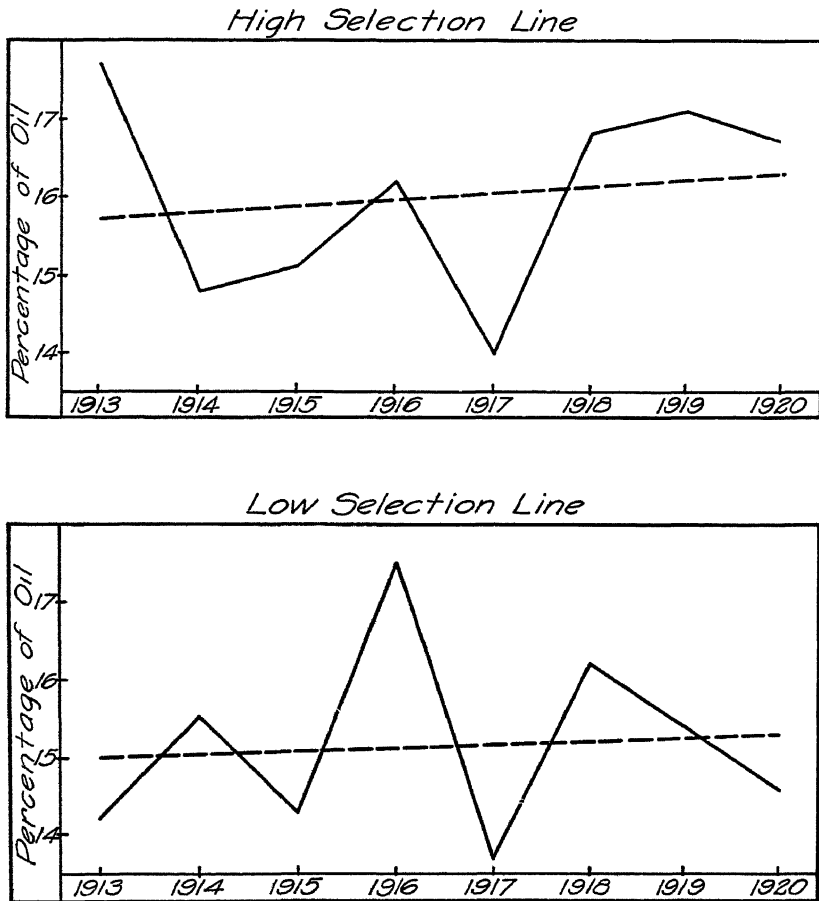


FIG 3.—Yearly averages of oil percentage in the high and the low selection lines of soy beans for a period of seven years (1913–1920). The broken line is a fitted straight line to indicate general trend

The average oil percentages for the eight years of selection work appear in Table 10 and are graphically presented in Figure 3.

A glance at Figure 3 shows that there has not been a marked change in the average oil production per plant as the iodine numbers have increased or decreased. In both lines there is a very slight increase in oil percentage during the eight years. The average percentage of oil in the high-line material during the last three years is approximately 16.8, while in that of the low line for the same period

it is approximately 15.4. In other words, there is a difference between the two lines of over 1 per cent.

The exact differences in oil percentage are shown in Table 11. The original plants that served as the beginnings of the high and low selection lines happened to show a marked difference in amount of oil ( $17.7 - 14.2 = 3.5$ ). This difference was not maintained during the early years of the experiment (1914-1916). But it is clearly evident that as the high and low lines began to draw apart in iodine number (beginning in 1916 or 1917), this difference in percentage of oil again became manifest. It is certainly significant in the last three years of the experiment.

TABLE 11.—*Difference between the average percentages of oil in the high and low selection lines*

Year	Selection line	Mean oil percentage	Difference	Year	Selection line	Mean oil percentage	Difference
1913	High	17.7	3.5	1917	High	14.0±0.1	0.3±0.1
	Low	14.2			Low	13.7±.1	
1914	High	14.8±0.1	-.7±0.1	1918	High	16.8±.1	.6±.1
	Low	15.5±.1			Low	16.2±.1	
1915	High	15.1±.2	.8±.3	1919	High	17.1±.1	1.7±.1
	Low	14.3±.2			Low	15.4±.1	
1916	High	16.2±.1	-1.3 .1	1920	High	16.7±.1	2.1±.1
	Low	17.5±.1			Low	14.6±.1	

The cause of the slight increase of oil percentage in both lines is difficult to explain. It may lie in the continued and unconscious selection for better plants, especially those with larger seeds and better yields. At least the experiment indicates that one may select for iodine value without decreasing the percentage of oil in the soy bean.

A verification of the above-noted relationship between percentage of oil and iodine number is afforded by a correlation study between these two characters. When the iodine values and percentages of oil are correlated the correlation coefficients do not indicate a noticeable relationship between these two characters.

TABLE 12.—*Correlations between percentage of oil and iodine number in soy beans*

From chemical analyses of—	Year grown	Correlation coefficient
Progeny of original plant	1913	-0.05±0.16
No. 17277	1913	+ .14±.09
No. 17277—duplicate	1914	- .20±.08
High-selection line	1916	+ .33±.09
Low-selection line	1916	+ .10±.15
High-selection line	1917	- .13±.08
Low-selection line	1917	- .06±.10
High-selection line	1918	+ .16±.09
High—duplicate seed of high 1918	1919	- .21±.13
Low-selection line	1918	- .01±.10
High-selection line	1919	+ .17±.09
Low-selection line	1919	+ .32±.05
High-selection line	1920	+ .12±.08
Low-selection line	1920	- .42±.15
Composite sample of high line	1917-1920	- .13±.04
Composite sample of low line	1917-1920	- .27±.05

Correlation coefficients were calculated for these chemical characters over a period of years. The high-line data were considered separately from the low line. Within the high line only the late tall type of plant was considered, since a close relationship between the plant type and the iodine value has already been proved. Hence, beginning in 1916, when the differentiation between the late tall and the early dwarf type was apparent, the high-line data include only the former. A summary of the correlation coefficients appears in Table 12.

The striking fact in this table is the variability of the coefficients of correlation. In slightly more than half of the cases they are negative, in the other half, positive. The same seed gives a positive correlation one season and a negative correlation another. In some of the years the correlations are statistically significant but of low degree. It is true that the number of observations in any one year is very limited, but with so many years' data it seems perfectly obvious that no constant relationship between iodine number and percentage of oil can be said to exist. An unweighted average of the negative coefficients gives a value of  $r = -0.16$ , while a similar average of the positive coefficients is  $r = +0.19$ .

If one were permitted to average the entire list of correlations, the result would show none of any significant value. The fact that the data of both the high and the low line, when all the chemical analyses are grouped, show a negative correlation which might be statistically significant, means very little actually. Such a composite sample is affected more by the yearly relations of the chemical analyses to one another than by any inherent relation between the two correlated characters. This is but another indication that the climatic factors of the different seasons are extremely potent in their influence on the oil of the soy bean.

Considering the fact that the trend of the percentage of oil was not apparently affected directly by the increased and decreased values of the iodine numbers as the years went on (fig. 3), together with the lack of consistent correlation between oil percentage and iodine number, it seems reasonable to conclude that these two characters are not very intimately related to each other. One can select for an increased quality of oil without decreasing the quantity of oil produced by the soy-bean plant.

## DISCUSSION

From the results of this investigation it seems clear that any improvement in the drying quality of soy-bean oil is not likely to come through continued selection in any one strain that has been purified by ordinary selection methods. There are two reasons for this. First, the ordinary variety or strain of soy bean is reasonably pure for its inherent oil characteristics. This is undoubtedly due to its method of self-pollination, which tends to isolate relatively pure (or homozygous) strains naturally, especially if the quality of the oil is correlated with a definite morphological or physiological character. Second, the quality of the oil (because of the nature and percentage of the unsaturated fatty acids) is readily influenced by external conditions (nonheritable), which tend to obscure any small, heritable differences that might exist and might be used in selection.

Accordingly, the quickest method of determining the best types of soy beans in respect to oil quality is to analyze soy beans of as many different varieties or strains as possible, and to select the highest-analyzing strain which is adapted to a specific region and which has proved to be a good yielder. Judging from all known reports, it is doubtful whether the iodine value of the soy bean can be raised to an average of 140. It may be that the highest average value now known, which is 134 to 135, represents nearly the physiological limits of the soy-bean plant. It is true that occasional analyses show values as high as 141 to 143, which might indicate further possibilities of the plant, but this is doubtful.

If selection methods are ineffective there remains the hope that hybridization may be effective in raising the iodine value. It is entirely reasonable to believe that by crossing two different varieties, one might eventually combine in the progeny certain favorable factors for oil quality absent in one or the other parental varieties.

As regards quality and quantity of oil, the experiments reported in this paper give strong evidence that there is little or no interrelation. In other words, selection for high quality will not necessarily depress to any extent the quantity of oil produced by the plant. One may reasonably hope to isolate a high-quality variety that will yield not only a good amount of seed, but also a high percentage of oil. For example, the writers' high-line strain, which shows an average iodine number of 133.7 (three-year average), is classed as a good yielder with an average percentage of oil of 16.9 (three-year average).

As a result of these selection experiments certain general conclusions seem worthy of discussion. It was noted that the continued selection for high-quality oil produced a late-maturing strain, while the continued selection for low-quality oil resulted in an early strain. Data on the critical time for the formation of the unsaturated acids that are responsible for the quality of the oil indicated a point very late in the life history of the plant—just preceding maturity. A possible deduction was made that, in view of the fact that a cool climate seems to favor an increase in the iodine number, the late strain was higher in quality because it entered the critical period for oil formation when the weather was cooler. The low-quality strain, being fully two weeks earlier, matured when the weather was warmer.

If this situation proves to be a general phenomenon, it deserves considerable attention. The soy-bean oil industry at the present time is located in the Southern States, where cotton mills are used for expressing the oil. Obviously, this situation may not be the most efficient and economical one. Although the soy-bean oil industry is in a pioneer stage—at least as far as production is concerned—the huge amount of imported soy-bean oil indicates a vigorous growth of the market. According to a letter received in 1921 from the Bureau of Chemistry of the United States Department of Agriculture, "the larger part of the soy-bean oil used in this country is imported from the Orient. In comparison with the imported oil and beans, the amount of oil produced from domestic beans is practically negligible." Chemical analyses prove that domestic oil is of as high quality as imported oil.

The iodine value of oil, because of its intimate relation to the drying quality, is the most important standard by which to judge oils

intended for use in paints. In view of the variations in iodine number noted in these investigations, it will be necessary ultimately to buy oil on the basis of a standard test for quality, just as milk is now judged by its butterfat content and not merely by its bulk. For a drier in paint manufacture it is absolutely essential that high quality of oil be uniformly present.

### SUMMARY

Continuous selection both for high and low quality of oil within one variety of soy bean for seven years produced a high and a low line differing to a significant extent in their average iodine number. An average of the last three years' data shows an iodine value of 133.7 for the high line and 124.9 for the low line. This is interpreted as the result of the isolation of two different genotypes within the original variety rather than of any change in a pure line.

The high line, selected entirely on the basis of chemical analyses, proved to be a late tall type with purple flowers, whereas the low line was early dwarf, and white-flowered. This was proof that the original commercial variety was neither a pure line for oil characteristics nor for season, growth habit, or even flower color.

Indirect evidence and some experimental data offer the interesting deduction that high quality of soy-bean oil is intimately correlated with a certain lateness of maturity. It is inferred that late maturity (in Wisconsin) provides the more favorable conditions for the complete development of the unsaturated acids that are responsible for high quality of oil.

Selection for high or low quality of oil proved to have no appreciable effect on the quantity or percentage of oil produced by the plant. This fact was verified by the lack of any consistent correlation between iodine number and percentage of oil. Correlation coefficients for these two characters taken over a period of years showed a high degree of variability but no consistent relationship. Such a situation makes it possible to select for high quality without decreasing the percentage of oil.

Large increases in the quality of soy-bean oil in any one variety by selection methods of breeding are not deemed possible.

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## GROWTH OF FRUITING PARTS IN *GOSSYPIUM CERNUUM*, AN ASIATIC COTTON<sup>1</sup>

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### INTRODUCTION

Studies on the growth of the fruiting parts of several varieties of American upland cotton (*Gossypium hirsutum*) and on Pima Egyptian and sea-island cotton (*G. barbadense*) have been reported in recent publications of the United States Department of Agriculture.<sup>2</sup> Although comparisons were made under a rather wide range of conditions, only slight differences were found in the rate of growth of the fruiting parts of the two types. Since the Asiatic cottons are in many respects widely different from both upland and sea-island, it seemed worth while to extend the comparison to include one of these.

*Gossypium cernuum* (Tod.), commonly known as Garo Hill or wool cotton, a native of India, is a species with so many divergent characters that differences in its habits of growth might reasonably be expected. Accordingly, a study of the growth of the fruiting parts of this species was begun at Greenville, Tex., in 1924. Data were recorded on the succession of fruiting branches, period of bud development, succession and growth measurements of floral buds, shedding of buds and young bolls, and growth measurements and maturation period of bolls. Comparable data were recorded for the Lone Star variety of upland cotton grown under the same conditions.

While the results of these studies indicate that Garo Hill differs but little in its growth periods from the American upland varieties or the American Egyptian and the sea-island varieties, as previously reported, the data are of interest as a further contribution to the available information on the fruiting habits of the cotton plant in relation to production under boll-weevil conditions. Some of the

<sup>1</sup> Received for publication Sept. 1, 1926; issued August, 1927.

<sup>2</sup> BALLARD, W. W., and SIMPSON, D. M. BEHAVIOR OF COTTON PLANTED AT DIFFERENT DATES IN WEEVIL-CONTROL EXPERIMENTS IN TEXAS AND SOUTH CAROLINA. U. S. Dept. Agr. Bul. 1320, 44 p., illus. 1925. McNAMARA, H. C., HUBBARD, J. W., and BECKETT, R. E. GROWTH AND DEVELOPMENT OF COTTON PLANTS AT GREENVILLE, TEX. U. S. Dept. Agr. Circ. 401, 18 p. 1927.

MARTIN R. D., BALLARD, W. W., and SIMPSON, D. M. GROWTH OF FRUITING PARTS IN COTTON PLANTS. Jour. Agr. Research 25: 195-208, illus. 1923.

Asiatic cottons are adapted to short-season conditions, but the bolls are too small for economical picking by hand. The bolls of the Garo Hill are the largest of the Asiatic series, and are of a size comparable with small-bolled upland varieties.

### SUCCESSION OF FRUITING BRANCHES

The appearance of the fruiting branches on the cotton plant has been described as follows: "The first indication of a new fruiting branch is the appearance of a minute triangular bud, commonly called a 'square,' deeply inclosed between the stipules of the primary leaf. The appearance of the square always precedes the development of the internode on which it is borne, and may therefore be considered as a definite indication of the formation of a fruiting branch."<sup>3</sup> This criterion was used in recording the appearance of fruiting branches on Garo Hill.

The interval between the appearance of successive fruiting branches on the main stalk was variable, ranging from 1 to 2 days early in the season, when the plants were making a rapid growth, to 8 or 9 days late in the season when plant growth was much slower. The mean interval was  $2.31 \pm .177$  days.

TABLE 1.—Interval between the appearance of successive fruiting branches on Garo Hill, Lone Star, and Pima cotton

Variety	Locality	Year	Mean number of days between appearance of successive fruiting branches
Garo Hill.....	Greenville, Tex.....	1924	$2.31 \pm .177$
Lone Star.....	do.....	1924	$2.84 \pm .077$
Pima.....	Sacaton, Ariz.....	1921	$2.81 \pm .043$

From the figures presented in Table 1, it can be seen that the mean interval between the appearance of successive fruiting branches produced on the main stalk of Garo Hill was about one-half day shorter than that recorded on Lone Star and Pima, but the probable error of the difference shows that it is not significant.

### SUCCESSION OF FLORAL BUDS

The date that each square appeared on the fruiting branch was recorded, and from these records it is possible to compute the number of days between the appearance of successive squares or buds on the fruiting branches. This interval was found to range from 3 to 12 days, with a mean of  $6.50 \pm .167$  days. Of the 196 cases recorded only 3 had an interval of less than 4 days and 8 over 10 days. There was no significant difference in this interval between Garo Hill and Lone Star or Pima. The mean number of days between the appearance of successive squares<sup>4</sup> on the fruiting branches of Garo Hill, Lone Star, and Pima are given in Table 2.

<sup>3</sup> MARTIN, R. D., BALLARD, W. W., and SIMPSON, D. M. GROWTH OF FRUITING PARTS IN COTTON PLANTS. Jour. Agr. Research 25: 195-208, illus. 1923.

<sup>4</sup> The term "appearance of square" is used to designate the time when the triangular fruiting bud becomes visible to the naked eye.

TABLE 2.—*Mean number of days between the appearance of successive squares on the fruiting branches of Garo Hill, Lone Star, Pima cotton*

Variety	Locality	Year	Mean number of days between appearance of successive squares on fruiting branches
Garo Hill.....	Greenville, Tex.....	1924	6.50±.167
Lone Star.....	do.....	1924	6.11±.107
Pima.....	Sacaton, Ariz.....	1921	6.35±.069

A tendency was noted in Garo Hill for the interval between appearance of successive squares on the fruiting branches to lengthen when plant growth was retarded late in the growing season. This is in agreement with results reported on other species of cotton.

## GROWTH MEASUREMENTS OF FLORAL BUDS

Daily growth of the floral buds was recorded by measuring the length from the base of the calyx to the corolla tip. The measurements were begun when the buds had been visible from 7 to 8 days, at which time they were 2 to 3 mm. There was a mean interval of 18 days from the date of first measurement until bloom, with only 10 deviations from the mean. The mean daily growth measurements of the floral buds on Garo Hill, together with those recorded on Lone Star in 1922,<sup>5</sup> are given in Table 3.

TABLE 3.—*Mean length of floral buds of Garo Hill and Lone Star from 18 days to 1 day preceding bloom*

Days preceding bloom	Mean length of floral buds of—		Days preceding bloom	Mean length of floral buds of—	
	Garo Hill, 1924	Lone Star, 1922		Garo Hill, 1924	Lone Star, 1922
	Mm.	Mm.		Mm.	Mm.
18.....	2.13		9.....	9.62	9.67
17.....	2.94		8.....	10.47	10.30
16.....	3.01		7.....	11.34	11.17
15.....	4.51	6.02	6.....	12.00	12.22
14.....	5.72	6.37	5.....	13.20	13.10
13.....	6.48	7.20	4.....	14.64	14.28
12.....	7.37	7.62	3.....	16.15	15.60
11.....	8.05	8.36	2.....	19.10	17.70
10.....	8.78	8.96	1.....	29.80	24.37

From these data it can be seen that the elongation of the average Garo Hill floral bud for the first 13 days on which measurements were made was about 0.8 mm. per day. During the next three days the increase was approximately 1.3 mm. per day. The second day preceding bloom the average elongation was 2.93 mm. The mean length of the floral bud on the day before bloom was 29.8 mm., or an increase of 10.7 mm. over the length on the second day before bloom.

<sup>5</sup> MARTIN, R. D., BALLARD, W. W., and SIMPSON, D. M. Op. cit.

This marked increase in the length of the floral bud on the day preceding bloom is due to the rapid enlargement of the corolla and inclosed staminal column.

Fifteen days before blooming the Garo Hill floral buds were somewhat smaller than those of Lone Star, but the daily increase in length was slightly more rapid than in Lone Star, so that by the ninth day

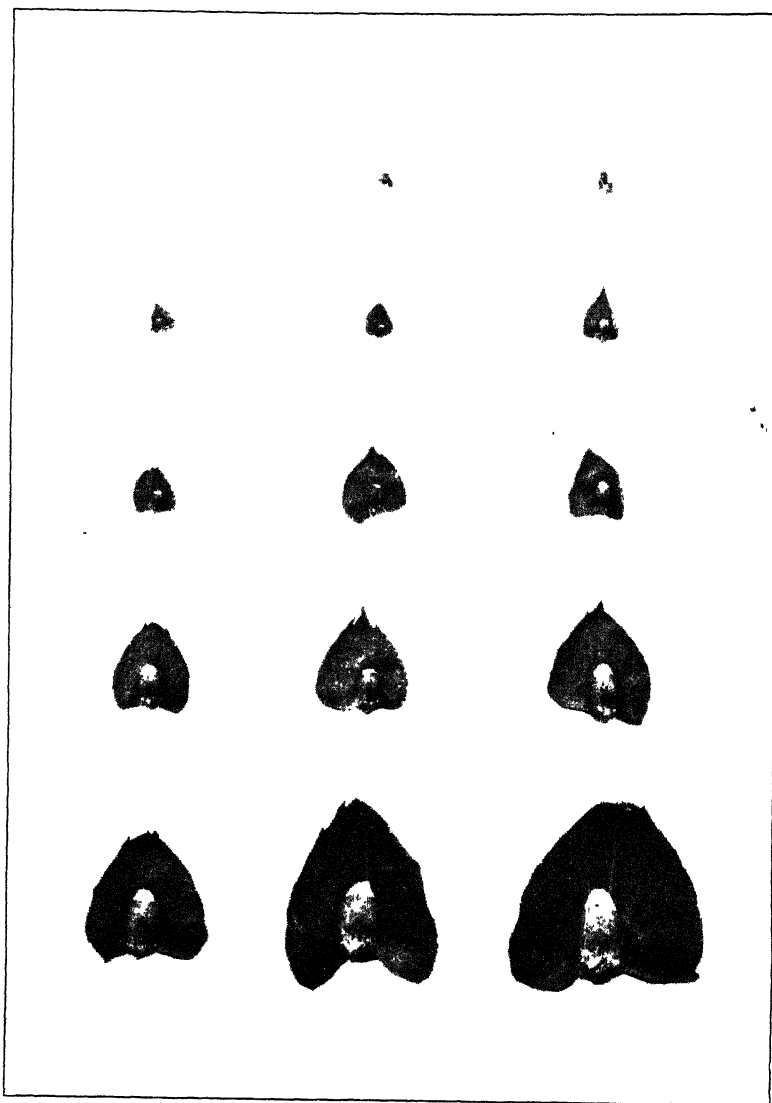


FIG. 1.—Floral buds of Garo Hill cotton (*Gossypium ecrnum*), Greenville, Tex., 1924, showing the size of the floral buds from 23 to 9 days preceding bloom. Natural size

preceding bloom the buds were nearly equal in length. On the second day previous to bloom the length of the average Garo Hill floral bud was 19.1 mm., and Lone Star was 17.7 mm. The day before flowering the Garo Hill buds increased 10.7 mm., and the

Lone Star buds 6.67 mm., so that the final mean lengths were 29.8 for Garo Hill and 24.37 mm. for Lone Star.

The daily growth of the floral bud of Garo Hill is illustrated in

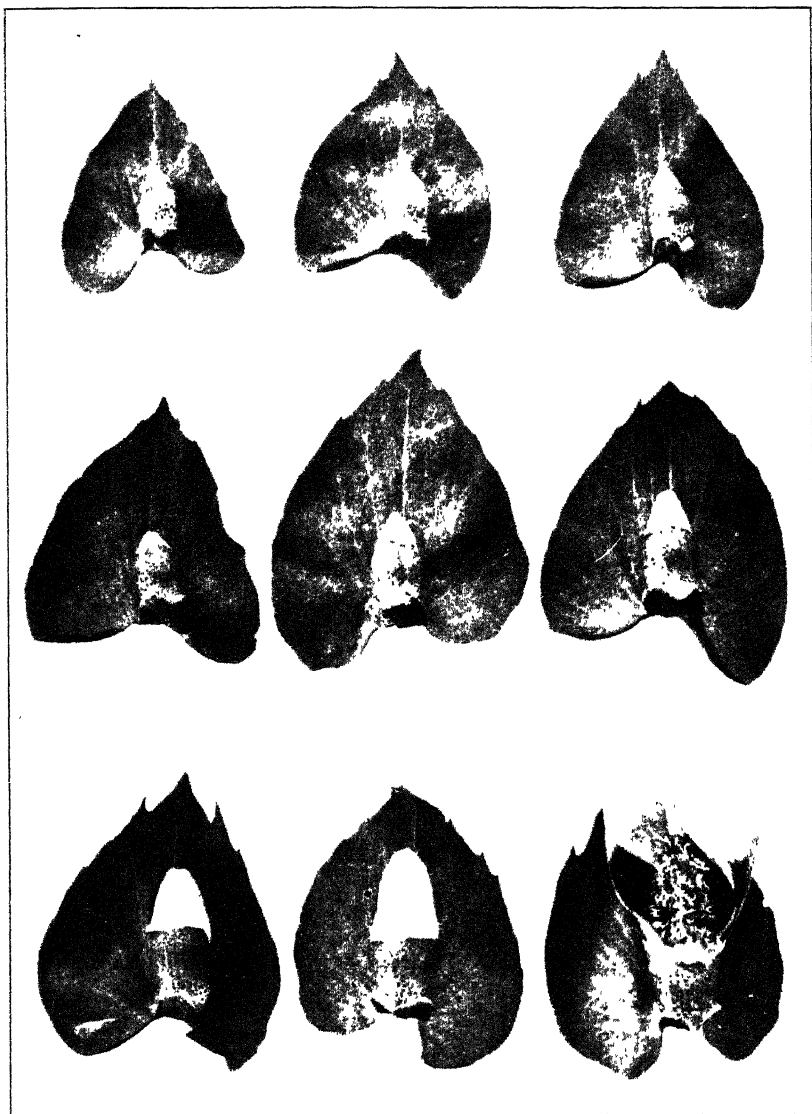


FIG. 2.—Floral buds of Garo Hill cotton (*Gossypium cernuum*), Greenville, Tex., 1924, showing the size of the floral buds from eight days preceding bloom until bloom.  $\times \frac{1}{15}$

Figures 1 and 2, which together show the size of the floral bud and one bract daily for 23 days preceding bloom. Figure 3 shows the size at 3-day intervals.

## PERIOD OF BUD DEVELOPMENT

The date that each bud appeared and also the date that each bud flowered were recorded, and from these dates the square period<sup>6</sup> was

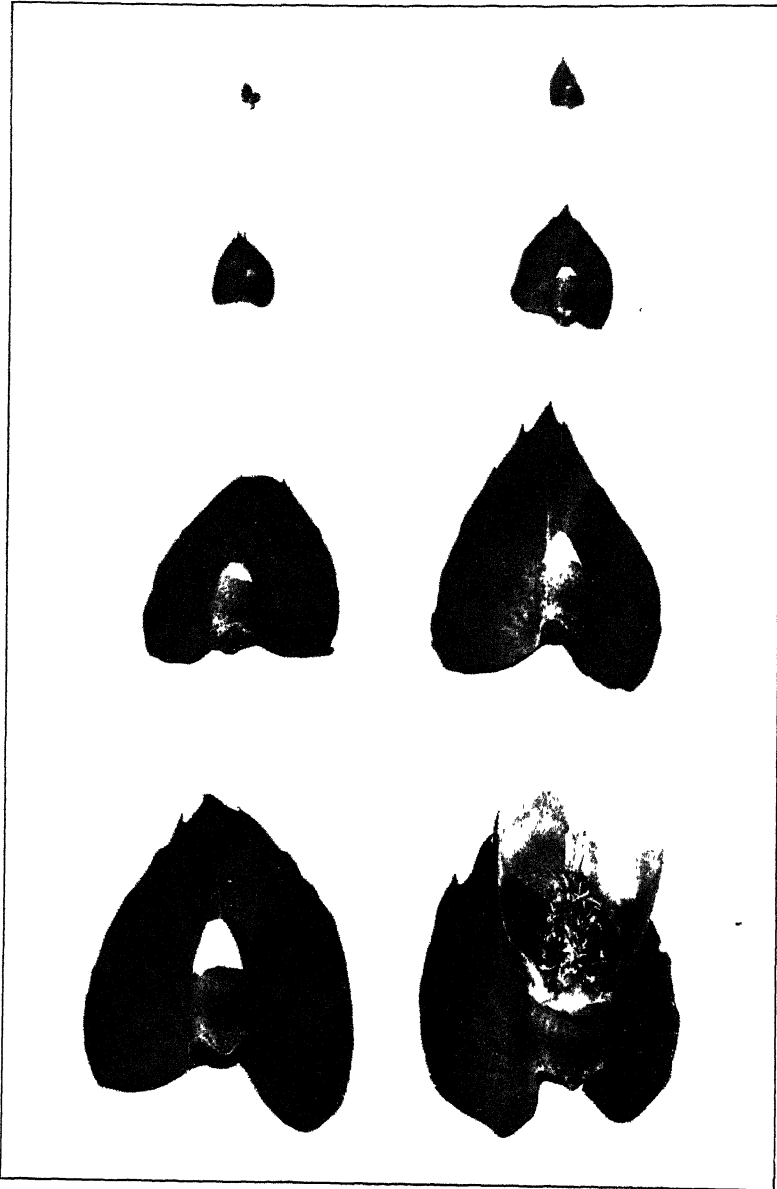


FIG. 3.—Floral buds of Garo Hill cotton (*Gossypium cernuum*), Greenville, Tex., 1924, showing the size of the floral buds at three-day intervals from 21 days preceding bloom until bloom. Natural size

determined. This period varied from 22 to 30 days, with a mean of 25.64 days. Buds produced late in the season, when plant growth

<sup>6</sup> The number of days from appearance of the bud until it flowers is referred to as the "square period."

had practically ceased, required a longer period than those produced earlier in the season. Only 4 of the 205 squares examined had a square period of 30 days, and 3 of these were on the topmost fruiting branches, while the fourth was on the outer node of a lower branch.

The mean square period for Garo Hill was  $25.64 \pm .32$  days, which was approximately the same as that recorded for Lone Star, namely,  $25.93 \pm .10$ . The square period recorded for Pima in 1921 was  $30.11 \pm .128$ . From these figures it is evident that the square period for Garo Hill and Lone Star was about 4.5 days less than for Pima.

#### SHEDDING OF BUDS AND YOUNG BOLLS

The interval from the appearance of buds to the date of shedding was very irregular both in Garo Hill and Lone Star, ranging from 2 to 18 days, with a mean of  $10.5 \pm .669$  days for Garo Hill and  $8.28 \pm .467$  days for Lone Star. There was a tendency in both cottons for the buds produced early in the season to shed in fewer days than those produced late in the season.

The number of days from flowering to shedding of bolls was very regular in Garo Hill, varying from 2 to 5, with a mean of 3.85. This period ranged from 1 to 11 days in Lone Star, with a mean of 6.10 days. These ranges are shown in Table 4, which gives the frequency distribution of the number of days from flowering to shedding of bolls for Garo Hill and Lone Star.

TABLE 4.—*Frequency distributions of the number of days from flowering to shedding of bolls on Garo Hill and Lone Star in 1924*

Variety	Number of bolls shed from 1 to 11 days after flowering, as indicated											Mean number of days
	1	2	3	4	5	6	7	8	9	10	11	
Garo Hill.....		2	25	35	17							3.85
Lone Star.....	1	1	4	10	15	20	18	11	4	2	1	6.10

The mean number of days from flowering to shedding of bolls was  $3.85 \pm .059$  days for Garo Hill. This was  $2.26 \pm .06$  days less than recorded for Lone Star, which was  $6.10 \pm .01$  days. Martin and Loomis<sup>7</sup> report that the mean persistence of shed bolls in Pima at Sacaton, Ariz., in 1920 was 10.8 days. This is about 7 days longer than that recorded for Garo Hill.

Whether it is a normal occurrence for the Garo Hill bolls to shed so soon after flowering or whether it was due to traumatic causes has not yet been determined. All of the bolls shed from the Garo Hill plants studied were carefully examined for insect injuries, and only five of the total shed appeared to be injured.

#### GROWTH MEASUREMENTS OF BOLLS

Data on the growth of the bolls were obtained by measuring the length, beginning the second day after flowering and continuing

<sup>7</sup> MARTIN, R. D., and LOOMIS, H. F. SUMMER IRRIGATION OF PIMA COTTON. Jour. Agr. Research 23: 927-946, illus. 1923.

daily for 20 days, at which time the bolls had attained their maximum length. The following record is based on measurements obtained on 176 bolls that reached maturity. The mean length of the bolls on the second day after flowering was 14.09 mm., and the mean daily increase in length was about 1.5 mm. for the next four days. Thereafter the growth rate was approximately 2.2 mm. per day until the bolls were 12 days old and had reached a mean length of 33.55 mm. The rate of growth after this age was attained was considerably slower, the bolls reaching a mean length of 37.67 mm. eight days later, which was an increase of only half a millimeter per day.

The growth rate on the Garo Hill bolls was similar to that recorded on Lone Star bolls at Greenville, Tex., in 1922.<sup>8</sup> The daily growth measurements on Garo Hill bolls are shown in Table 5.

TABLE 5.—Mean length of bolls of Garo Hill from 2 to 20 days after flowering

Days after flowering	Length of bolls	Days after flowering	Length of bolls
	<i>Mm.</i>		<i>Mm.</i>
2.....	14.09±0.178	12.....	33.55±0.276
3.....	15.73±0.198	13.....	35.44±0.255
4.....	17.18±0.292	14.....	36.52±0.278
5.....	18.36±0.265	15.....	37.05±0.247
6.....	20.57±0.264	16.....	37.35±0.264
7.....	23.00±0.249	17.....	37.51±0.356
8.....	24.50±0.320	18.....	37.63±0.291
9.....	27.15±0.295	19.....	37.67±0.306
10.....	29.33±0.212	20.....	37.67±0.306
11.....	31.65±0.400		

The increase in size of the bolls is illustrated in Figure 4, which shows the size of the bolls every 5 days from bloom until 25 days after.

#### MATURATION PERIOD OF BOLLS

The period of maturation of Garo Hill bolls was found to vary from 39 days for bolls set early in August to 56 days for those set in September. The maturation period of bolls set at four 6-day periods from August 9 to September 1, is shown in Table 6. The lengthening of this period as the cotton plants reached their full growth was also found in Lone Star at Greenville, Tex., in 1924, which varied from 37.2 days for bolls set early in July to 53.7 days for those set in August. King<sup>9</sup> reports that the maturation period of Pima bolls at Phoenix, Ariz., in 1919, varied from 54 days for bolls set in July to 80 days for those set in September.

TABLE 6.—Maturation period of bolls of Garo Hill set at four 6-day intervals from August 9 to September 1, 1924

Maturation period of bolls set—	Days
August 9-14.....	40.67
August 15-20.....	44.31
August 21-26.....	50.78
August 27-September 1.....	52.60

<sup>8</sup> MARTIN, R. D., BALLARD, W. W., and SIMPSON, D. M. Op. cit.

<sup>9</sup> KING, C. J. WATER-STRESS BEHAVIOR OF PIMA COTTON IN ARIZONA. U. S. Dept. Agr. Bul. 1018, 24 p., illus. 1922.

The mean maturation period of all Garo Hill bolls set was  $45.49 \pm .271$  days. This was 4.66 days longer than that recorded for Lone Star in 1924 and 22.51 days shorter than that recorded for Pima.<sup>10</sup>

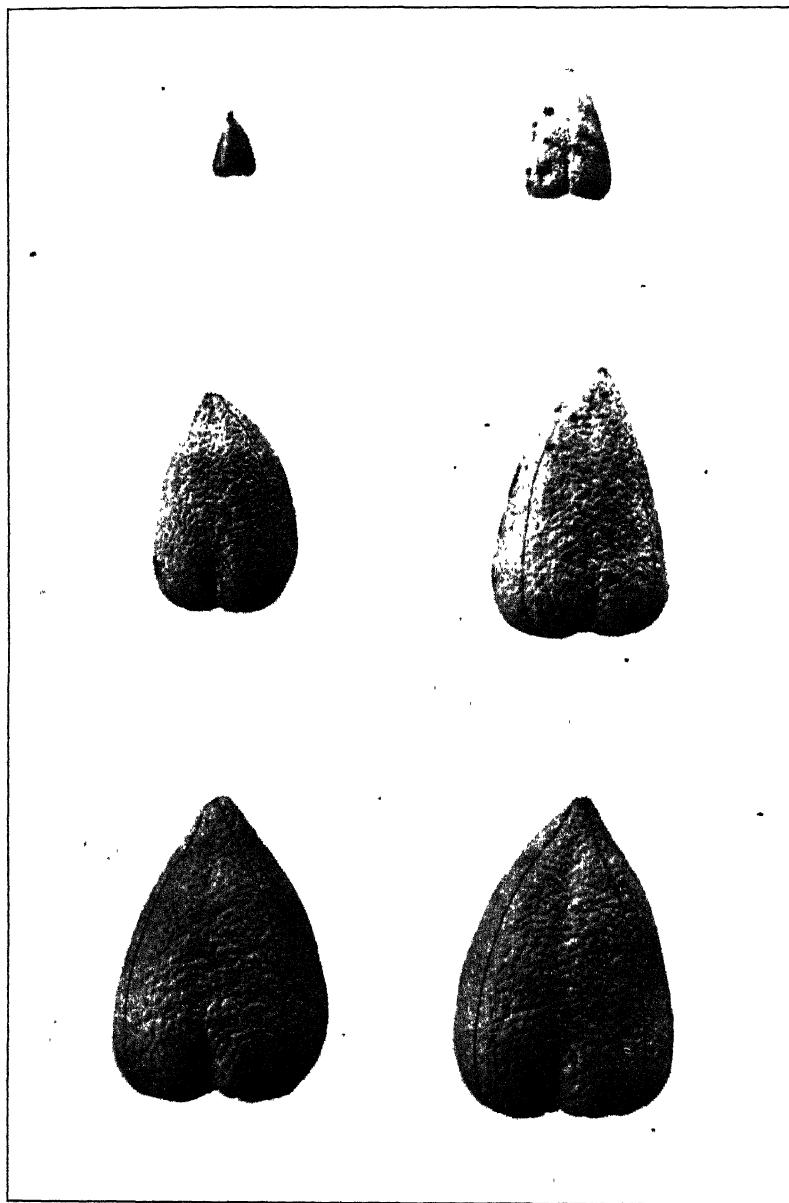


FIG. 4.—Bolls of Garo Hill cotton (*Gossypium cernuum*), Greenville, Tex., 1924, showing sizes of bolls at five-day intervals, from the flower to the adult size at 25 days. Natural size

The mean maturation period of Garo Hill, Lone Star, and Pima is presented in Table 7.

<sup>10</sup> KING, C. J. Op. cit.

TABLE 7.—Mean maturation period of bolls of Garo Hill, Lone Star, and Pima

Variety	Locality	Year	Mean maturation period
			Days
Garo Hill.....	Greenville, Tex.....	1924	45 49±. 271
Lone Star.....	do.....	1924	40 83±. 178
Pima <sup>a</sup> .....	Phoenix, Ariz.....	1919	68. 00

<sup>a</sup> KING, C. J. Op cit

## SUMMARY

The Garo Hill cotton (*Gossypium cernuum*) is one of the Asiatic series which is very remote in plant characters from the Egyptian and upland varieties grown in the United States, so that differences in habits of growth might be expected. Most of the Asiatic cottons are not adapted for cultivation in the United States on account of their very small bolls, but the Garo Hill has the largest bolls of any Asiatic type; nearly as large as those of the Texas big-boll cottons, as represented in this comparison by the Lone Star variety.

The growth rates and sequence in development of the fruiting parts of the Garo Hill cotton were determined at Greenville, Tex., by the same methods that were used on the Egyptian and upland types of cotton, and reported in previous publications.

The interval between the appearance of successive fruiting branches on the main stalk is somewhat shorter in the Garo Hill cotton than in the other types, while the interval between successive squares on the branches is somewhat longer. The shedding of abortive bolls in Garo Hill occurred more promptly, within 3 to 5 days after flowering, while many abortive bolls of Lone Star were held for 6 to 8 days, and some from 9 to 11 days. The rate of growth of the buds and bolls was nearly the same in the Garo Hill cotton as in the other types. The maturation period of bolls of the Garo Hill was somewhat longer than that of Lone Star, though the difference was slight and may not be significant.

In the latter part of the season a lengthening of the maturation period of Garo Hill bolls was found, which also is in agreement with the behavior of the Lone Star and Pima varieties. Garo Hill bolls which were set early in August had a period 10 to 12 days shorter than bolls set in September.

# THE BEHAVIOR OF THE ANTHOCYAN PIGMENTS IN CANNING

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## INTRODUCTION

Many fresh fruits owe a considerable part of their attractiveness and consequent popularity to the bright red and purple colors of the anthocyan pigments present in the skin or flesh. It is desirable that the canned products made from these fruits retain the same brightness and freshness of color seen in the raw material. In the case of strawberries, raspberries, and cherries the degree to which the canned product retains the brilliance of color of the fresh fruit largely determines the desirability of a variety for canning purposes.

Much difficulty is encountered in securing satisfactory preservation of the natural colors in these and in many other fruits when canned, especially when the canning is done in tin. As a means of preserving more of the original color, the canning industry has resorted to the use of cans specially prepared by coating the inside with a lacquer designed to prevent contact of the contents with the metal. While the lacquered can permits much better preservation of color, it has proved a serious disappointment in another respect, as rapid corrosion of the metal occurs at breaks in the coating, and perforation of the can takes place much earlier than when plain tin is used. In consequence, the canner is forced to choose between placing heavily pigmented materials in plain tin, thereby losing in appearance but gaining in length of time over which the product may be held before consumption, or in lacquered cans, thereby gaining in appearance of the product but decreasing the time which will elapse before loss from perforation occurs.

A rather careful search of the literature has failed to reveal any detailed studies of the behavior of the anthocyan pigments in the course of canning processes. Various authors have mentioned the fact that fading or partial destruction of these pigments occurs, but they have advanced no suggestions as to the causes for the changes. The fact that pink, violet, or purple discolorations sometimes occur in peaches, pears, and other fruits has also been noted, but no investigations of the causes of such discolorations appear to have been made. Various tentative explanations have been offered, among which over-processing is most frequently mentioned. Among other causes, the use of sunburned fruit (8),<sup>2</sup> of fruit grown in very hot localities (10), permitting fruit to become overheated during shipment and before processing (8, 10), variety and degree of ripeness (6), and failure to cool promptly after processing (16), have been suggested. It has therefore been quite generally recognized that violet or pink discolorations are of occasional and sporadic occurrence in various

<sup>1</sup> Received for publication July 3, 1926; issued August, 1927.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 130.

canned fruits, but the reasons for such discolorations are very imperfectly understood, and it does not appear to have been suggested that the anthocyan pigments may stand in causal relationship to many cases of discoloration of this type.

The very extensive investigations of the anthocyan pigments made by Willstätter and his students (34, 36, 37) have shown that a single pigment may exhibit colors ranging from red to violet according as it is present in the free condition or as a salt. These workers have presented considerable evidence to show that many of the anthocyan form complex salts with metals, resulting in alteration in color. Shibata, Shibata, and Kasiwagi (32) have shown that in a very large number of anthocyan extracted from the colored parts of plants the addition of a metal to the aqueous solution produces a shifting of the color toward the violet end of the spectrum, the degree of alteration in color depending upon the metal employed. With aluminum sulphate and stannous chloride the colors produced, regardless of the original color of the pigment solution, are described as violet-red, pink, indigo, or lilac. That is, the action of either of these metals resulted in the production of some degree of violet color as a consequence of partial or total conversion of the free pigment into the violet-colored metallic salt. Nikiforowsky (27) considers that the color reaction with aluminum chloride is a specific reaction for anthocyan which permits them to be distinguished from flavonols and tannins. It is therefore well established that anthocyan generally, regardless of the color of the free pigment, react with many metals with an accompanying alteration of color toward the violet end of the spectrum.

It will be shown in the present communication that the presence in the material of anthocyan which react with the metal of the can is responsible for the discolorations characteristic of many fruits when canned in tin, and that the reaction of the pigment with the metal of the can is also largely responsible for the corrosion and perforation so characteristic of many of the pigmented fruits.

#### ANTHOCYAN PIGMENTS IN RELATION TO PINK OR PURPLE DISCOLORATION

In a number of fruits the anthocyan color is localized chiefly in the skin, and as a consequence of peeling is not usually present in the canned material. This is the case with the peach, the canned fruit usually showing no red or purple color. Any pink or violet discoloration which develops in the material subsequent to canning is therefore considered abnormal and often results in the discarding of the fruit as unwholesome and possibly poisonous.

A rather general appearance of discoloration in peaches put up by home canning clubs occurred in 1924 over a limited area lying between Athens and Augusta, Ga. Nearly all peaches canned in tin developed some degree of color, ranging from light pink to deep purple. In consequence, canning in tin was suspended, the trouble being popularly attributed to some defect in the tin cans being used.

The situation was brought to the attention of the writers by L. V. Strasburger, a chemist representing one of the can-manufacturing companies, who had spent several weeks in the territory in an attempt to find means of eliminating the difficulty. Strasburger

submitted a number of cans of material for examination, and described his experimental work in detail. He had employed a great variety of modifications in time and temperature of exhausting and of processing, in concentration of sirup used, and in degree of ripeness of fruit employed, but more or less purple discoloration was invariably present in his experimental packs. Substitution of hand peeling for lye peeling gave no better results. Tin cans from several different makers were employed, some of which had been left from a supply used with satisfactory results in previous years. No differences were noted, however, in the material in the different cans, color appearing in all in about the same degree. In glass, the fruit was in every case a perfectly normal, clear yellow color. As this suggested that the discoloration was produced by tin, Strasburger processed fruit in glass jars to which metallic tin or stannous chloride had been added. Discoloration identical with that occurring in cans resulted. At this point, Strasburger presented the case to the writers, stating his conclusion that the discoloration was due to a reaction of some constituent of the fruit with the metal of the can, and asking their assistance in determining the exact nature of the trouble. A similar request was subsequently made by a number of canning club leaders and home demonstration agents. In consequence, the writers undertook to determine the class of compounds in the peach responsible for the trouble, subsequently expanding the work to include a considerable number of other fruits.

#### EXAMINATION OF PEACHES IN 1924

Material of the variety Belle of Georgia, canned by Strasburger at Athens, was subjected to careful examination upon its arrival at the laboratory. The riper pieces showed considerable bluish purple discoloration at the surface, and adhering bits of peel at the spray-burned tips were bluish in color. The firmer hard-green fruits were usually normal in color but occasionally were faintly violet. Purplish discoloration in some degree was evident in the stone cavity of almost every piece, but was much more pronounced in the riper fruits. The interior surfaces of the cans showed some corrosion but this was not excessive, being less than that found in standard commercial canned peaches purchased and opened for purposes of comparison. As it was noted that the discoloration apparently deepened in tint after the cans were opened, the fruit was emptied into shallow porcelain dishes and allowed to stand at room temperature overnight. The color was greatly intensified, the riper pieces becoming uniformly purple, while the liquid became so deeply purple as to appear almost black.

This condition recalled an observation made earlier in the season. In the course of a visit to some of the canning plants in the vicinity of Fort Valley, Ga., the writers had examined samples of unpeeled pie peaches showing a somewhat similar discoloration. The fruit, which was somewhat underripe, was merely washed with warm water after stoning, packed in the cans, and given the usual processing treatment. When some of these cans were opened it was observed that the bright red color of the skins had faded to a dull purple or muddy violet, and that the liquid in the can was a cloudy purple. This was recognized as an alteration of the skin pigments, and led

the authors to suspect that the discoloration observed in the peeled fruit might be due to the same cause.

Analyses made in the Bureau of Chemistry showed that the tin content of the discolored fruit, while somewhat in excess of that usually present in peaches examined so soon after packing, was not greater than that usually found in commercially canned peaches six to nine months after canning. Consumption of large amounts of the material by the writers showed that it was without harmful effects.

Some experiments designed to test the hypothesis that the development of purple discoloration was due to alteration in the anthocyanins of the fruit were made on August 29, 1924, employing Elberta peaches grown at Arlington Experiment Farm, Rosslyn, Va.

The fruit was separated into two lots, one of which was soft ripe, the other in prime canning condition. The fruit was peeled by hand, packed in No. 2 plain tin cans, given a light exhaust, and processed 15 minutes at 100° C. A part of the material was packed with distilled water, a part with 30 per cent sirup, and a part with 30 per cent sirup to which 1 per cent of sodium chloride had been added. All the material was normal in color when opened except for a very slight purpling in the stone cavity in the case of the riper fruits packed in water or in sirup to which salt had been added. No change in color occurred when the material was allowed to stand in air overnight. The peels and stones from the fruit were packed in cans separately, water added, and processed exactly like the fruit. On opening, the peels showed different degrees of purpling corresponding to the degree of ripeness. The most intense color developed in peels from the ripest fruit, and the color became very intense on standing in air. The stones were intensely purple when the cans were opened. Additions of sodium hydroxide and ammonia in different amounts prior to canning decreased the discoloration of peels somewhat but did not wholly prevent it, and the material became deeply discolored on prolonged standing in air. Control lots of peeled fruits, peels, and stones were processed in glass. The fruit was normal yellow in color, the stones and liquid deep cherry red, while the color of the peels largely disappeared, the liquid becoming light red.

A quantity of stones were cracked, the kernels removed, and kernels and broken bits of stone were added separately to cans of peeled peaches prior to processing. The fruit with kernels added was normal except for a slight cloudiness of the liquid; that with broken stones had a deep purple liquor and the pieces of fruit were dyed superficially with the color.

These experiments pointed definitely to the red pigment as responsible for the discoloration, but indicated that contact with tin and subsequent exposure to air was necessary for its maximum development. They indicated also that the degree of discoloration depended directly upon the pigment content of the material. In consequence, some seedling fruit having a very intense red color extending from the skin into the flesh was employed for further work. Flesh, peels, and stones processed in tin showed considerably greater purpling at opening than did the Elberta, and on standing overnight in air the flesh became a royal purple.

An aqueous extract of the pigment of the stones was made by heating a quantity of stones in water at 90° to 100° C. for 15 minutes. This yielded a deep wine-red solution. Heating with granular tin or adding dilute stannous chloride to this solution resulted in change of the color to very dark purple. Addition of citric acid to give a final concentration of 1 per cent preserved the red with very little change, and smaller amounts retarded the change in color to a degree proportional to the concentration.

From these experiments it seemed clear that the anthocyan of the skin and stone were responsible for the discoloration, and, as the pigment of the peach has never been investigated, it appeared advisable to make at least a partial purification of a quantity of it for a further study of its properties.

#### SEPARATION OF THE PIGMENT

About 3 kilograms of broken peach pits from which the kernels had been removed were extracted five times with glacial acetic acid. The extract was concentrated to small volume at 40° C. under greatly reduced pressure. The pigment was precipitated as a heavy sirup by the addition of about 10 volumes of ether, and the precipitate was washed several times with ether. It was then saturated with NaCl and extracted with butyl alcohol, repeated extraction being necessary to remove the bulk of the pigment. The butyl alcohol was washed several times with saturated NaCl, followed by three washings with a small amount of distilled water. The butyl alcohol was then concentrated under reduced pressure at 40° and the alcohol allowed to evaporate further at room temperature. The larger part of the pigment was precipitated from the butyl alcohol by the addition of several volumes of ether, collected on a filter, washed repeatedly with ether, and dried over calcium oxide. No attempt was made to crystallize or further purify the bright red powder. Its solution in water was almost neutral and was deep red with a tinge of purple. When the solution was rendered faintly acid, the color became deep bright red. Ferric chloride in nearly neutral solution gave a bluish black color in dilute solutions and a bluish black precipitate in greater concentrations. When the solution was made slightly acid, ferric chloride gave a purplish color, deepening to black. The absence of tannins, which would give a green color in acid solution with ferric chloride, was therefore indicated.

When the nearly neutral solution was heated with granular tin metal, slight darkening occurred, but there was no appreciable alteration of the red color. An acidified solution heated with metallic tin gave a deep purple color. With increase in the acidity of the solution the purpling decreased, practically no change occurring in strongly acid solution. When stannous chloride was added to a neutral solution, a blue precipitate was obtained. If the solution was slightly acidified and stannous chloride added, it developed a purple color, and a purple precipitate was thrown down. With increasing degrees of acidity the purpling decreased, strongly acid solutions remaining red with a faint tinge of purple. Aluminum chloride threw down a greenish blue precipitate from neutral solution; in slightly acid solution no change in color occurred. Lead acetate gave a greenish blue coloration in neutral solutions, but was without apparent effect upon

a solution acidified with acetic acid. Zinc chloride gave a green color in neutral solution, which returned immediately to red when the solution was made slightly acid.

From the results of this examination of the pigment it is apparent that the purple discoloration observed in peaches canned in tin is due to a reaction between the tin of the can and the anthocyan pigment present. It is possible that iron may be a contributing factor, as the pigment shows somewhat similar color reactions with salts of iron. The results also suggest that the acid relations are quite important, since the degree of acidity greatly modifies the intensity of the color.

The explanation of the fact that discoloration of the canned product is occasional and sporadic in its appearance, lies in the fact that the amount of the pigment developed in the fruit is determined by climatic conditions, hence varies considerably from year to year. In the district about Athens and Augusta, Ga., in which it was observed in 1924, the summer had been exceptionally hot and dry, with more than normal sunshine, and all varieties of peaches were unusually highly colored. In the Fort Valley district of Georgia the season of 1924 was close to normal in temperature and sunshine, and the commercial canners encountered little or no discoloration. The season of 1925 in the Fort Valley district was one of considerable excess of sunshine and rather high temperatures, and the fruit of all varieties showed exceptionally brilliant color. As a consequence, considerable violet or purple discoloration was observed in the commercial pack of several canneries in that district. It is clear that seasonal conditions determine the occurrence of the trouble by controlling the amount of anthocyan pigment developed in the fruit.

#### EXPERIMENTS WITH OTHER FRUITS

The work with the peach was considered as definitely establishing the fact that purple or violet discoloration of the canned product is due to a reaction of the anthocyan pigment of the fruit with the tin of the container. As somewhat similar discolorations had been observed by the writers in grape juices canned in tin, as well as in certain fruits, they were led to believe that the phenomenon might be of somewhat general occurrence in the more heavily pigmented fruits. In consequence, experiments were carried out with a considerable number of such fruits, including grapes, plums, red and black raspberries, blackberries, strawberries, cherries, mulberries, apples, fruits of *Crataegus* and *Viburnum*, Indian strawberries, and with beets and red cabbage. The results obtained with these materials will be briefly described. In all cases such an amount of material was prepared as permitted examination a few days after canning and again after six to nine months' storage.

#### GRAPES

Uhland, a greenish yellow variety, was canned both in glass and tin. The two lots were indistinguishable in appearance when opened, both being a dull grayish green. No change occurred on standing in air. Catawba and Rogers No. 13, both of which are red varieties, placed in plain tin cans and processed without exhaust-

ing, were, respectively, deep reddish purple and faintly purple when opened. On standing 48 hours in porcelain dishes the color of the liquid from tin containers became intensely purple and the flesh of the berries was dyed by it. In glass jars the color was a faint clear red which did not alter on exposure to the air. Four black grapes—Cloeta, Eumelan, Worden, and an unidentified variety—differed only in the intensity of the deep purple color developed in tin. In glass all were purplish red or brownish red, but when the liquid was removed and diluted with water it was seen to be a clear red with no suggestion of purple. Berries and skins of the deeply pigmented varieties were separated and processed separately in glass and tin. The berries in glass and tin were the same grayish green. Skins processed in glass were a deep wine red and neither skins nor liquid changed color on exposure to air. A portion of the wine-red liquid, when heated after the addition of a strip of tin foil, became light purple and on cooling deepened to intense purple. Skins in tin cans were a magnificent royal purple when opened, and the liquid drained from them, when added to the normally greenish gray pulps, stained them purple. When a few skins of one of the black varieties were added to a can of Uhland, the greenish yellow variety, before processing the fruit became a brownish purple.

After standing in storage 8 to 10 months, plain tin cans containing red or black grapes were intensely corroded. In the grapes canned without separating pulp and skins, the skins had faded to a dull buff color, but the flesh of the berries was dyed purple from the surface inward and broken berries were colored purple throughout. The liquid was distinctly purple and quite milky in appearance, apparently from the presence of hydrous stannous oxide. On exposure to air the milkiness increased and the purpling of both fruit and liquid became very intense.

The behavior of the grapes canned in tin is in many points identical with the disorder of wines known as blue casse. This condition is frequently encountered, especially by the French wine makers, in red or white wines which have been brought in contact with iron at some stage of the process of manufacture, usually in pressing. The trouble usually becomes apparent only after the wine is aerated, as in racking. In the case of a deep red wine, such as that made from Lenoir or other heavily pigmented grapes, the color gradually changes after racking through bluish red to dull violet, with a thin iridescent film at the surface. Ultimately a blue-black precipitate settles out, leaving the wine more or less altered in color. In white wines, the color changes pass through grayish or leaden shades to dull brown or brownish black. There is an enormous literature upon blue casse which will not be reviewed here. The essential facts thus far developed are that blue casse is the result of the presence of iron, either in the fruit or derived from the equipment employed in pressing and handling the juice, and that the iron combines with some constituent of the wine, subsequently producing abnormal color and going more or less completely out of solution upon exposure to air. It is significant that red and white wines, which may be made from the same variety of grapes, show differences in the colors developed. In the case of red wines, the fruit is crushed and fermentation is allowed to begin before pressing in order that a considerable part of

the pigment of the skins may be brought into solution. In white wines, heavily pigmented varieties may be employed, but the grapes are pressed cold immediately after crushing, in order that the pigments may not be carried into the wine. The differences in color of the red and white wines which develop casse may be due to the fact that in the red wines iron reacts chiefly with the pigment in solution, while in white wines the reaction is mainly with tannins.

A somewhat similar condition has been noted by Donauer (11) in the manufacture of Concord grape juice. He states:

Concord grape juice is changed considerably in color by copper and copper alloys and nickel. The color is entirely destroyed by zinc or iron. Tin turns the color to a violet. Aluminum and its alloys or silver-plated metal are very satisfactory for such grape juice as far as its color is concerned.

The writers have found that the juices of any deeply pigmented grapes such as Lenoir, Clevenor, Concord, Clinton, and Eumelan, whether fresh or pasteurized, give immediate color reactions upon the addition of ferric chloride, stannous chloride, or aluminum chloride in the cold, or by heating with metallic iron or tin. Upon the addition of stannous chloride the color is immediately transformed to purple or violet, according as smaller or larger amounts of the chloride are added. When acid is added the color returns toward red, finally becoming indistinguishable from the original juice. With aluminum chloride the change of color is less pronounced and the amount of acid required to produce return of the original color is much less. Ferric chloride produces an intense purplish black coloration, but the original color returns if sufficient acid is added. It may be pointed out that an identical treatment is employed with red wines which have developed blue casse, as they are acidified with citric acid and aerated, which restores much of the original color. There can be little question that reaction of the anthocyanins with metals is responsible alike for the alterations in color and unfermented juices, in pigment-containing wines, and in the entire fruits when the materials are permitted to come in contact with metal.

#### APPLES

Three varieties of apples were employed. Two of these, Hyslop and Small Red Siberian, are crab apples having intensely red coloration in the peel with white or light yellow flesh. The third, Baxter, in addition to having a deep red skin has the flesh more or less colored by red streaks extending inward almost to the core line. Small Red Siberian was canned whole in tin and in glass. In glass the red pigment dissolved in the water and gave a reddish solution, the fruits becoming clear yellow. In tin both fruit and liquid were faintly purple and became intensely purple on standing, the staining effect of the liquid upon the flesh being especially pronounced. The fruits of Hyslop were peeled by hand, the peels themselves being canned separately. The peeled fruit showed no change in the light yellow color during processing or after opening and exposing to air; peels and liquid become quite purple during processing and were deeply colored after overnight exposure to the air. The red striping in the flesh of Baxter faded almost completely in the material canned in glass; in the material in tin it went over to a rather faint purple which had diffused into the surrounding flesh. After 24 hours' exposure to the air, the color deepened to a decided purple in both liquid and flesh.

## CRATAEGUS BERRIES

Fruits of red haw processed 20 minutes in No. 2 cans had the color discharged, the fruits becoming bright yellow and the liquid very faintly red. On exposure to air the liquid became faintly reddish purple. In glass jars the fruit was yellow and the liquid clear, remaining unchanged after prolonged exposure to the air.

## VIBURNUM BERRIES

Fruits of *Viburnum* sp. lost somewhat of their bright red color in processing and developed considerable purple on prolonged exposure to the air, but the development of the discoloration was much less rapid than in grapes, apples, or plums. Another sample of fruit which was greatly overmature and badly shrivelled, lost practically all color in processing and did not change to purple on standing.

## PLUMS

Only one variety of plum, the Abundance, was used. The bright red-skinned fruits were canned whole. Both fruit and liquid were intensely purple when the cans were opened and the color deepened to purplish black, the change being more rapid than in any other of the various fruits tested.

## RASPBERRIES

Three varieties of raspberries were employed. These were the St. Regis, a variety having bright clear red fruits; the Cuthbert, a dark red; and an unnamed black variety closely resembling the wild black form. These were exhausted 2 minutes and processed 45 minutes at 85° C. In glass the St. Regis retained its bright red color. In tin it was very similar in appearance to the samples in glass when first opened but on exposure to air the liquid developed a reddish purple color, and the fruits above the liquid became a very dark purple.

The Cuthbert and the black variety were canned in both plain and enameled tin, with controls in glass. The Cuthbert in glass was a clear dark red. The fruit in enameled cans showed considerable fading of the original color and had a distinctly purplish tint when first opened. After exposure to the air it went over to a purplish red distinctly darker than that of the fruit in glass. The loss of color was much more pronounced in plain tin than in enameled cans, and there was more purple apparent on opening. On standing the color became dark purplish red like that of the enameled cans.

The black raspberry underwent very little change in color in glass. In enameled tins the color was only slightly less good than in glass, but had a faint purple tinge. In plain tin the original color was largely replaced by deep purplish red. On standing in air the fruit from both enameled and plain tins became so intensely purple as to appear almost black.

## OREGON EVERGREEN BLACKBERRY

The color in glass and in enameled tin was deep dark red, the latter having a purplish tinge which was not present in glass. This increased only very slightly after 24 hours in the air. In plain tin the original color was markedly faded and the liquid was purple and milky, probably from the presence of hydrous stannous oxide, as the metal was greatly etched. After standing overnight the liquid was purplish black, with milkiness still persisting. A similar condition was observed in strawberries, cherries, cranberries, raspberries, and mulberries.

## STRAWBERRIES

The variety of strawberry used was the Klondike, the treatment employed being a 2-minute exhaust and a process of 30 minutes at 85° C. In glass and enameled tin the color faded to an unattractive brownish red, the fruit in cans having a purplish tinge not present in that in glass. In plain tin the color was a dirty brownish red and the liquid had a turbid, milky appearance, with slight purpling. Very little change in color occurred in fruit from either plain or enameled cans on standing overnight in shallow porcelain dishes.

In connection with another study in the laboratory, the writers had canned small experimental packs of about 220 selections and strains of strawberries representing most of the commercial varieties. In this work both plain and enameled cans were employed. Examination of this material eight months after its preparation shows a very wide range of behavior. The only generalization which appears to be justified is that the use of enameled cans in all cases contributes in some degree to preservation of the original color of the material. Varieties differ widely in their behavior. In some cases the original red color is replaced by varying shades of brown or brownish red, without suggestion of purpling either before or after exposure to the air. In other cases some degree of browning, varying from slight to extreme in the different varieties, was accompanied by discoloration which ranged from barely visible violet to intense purple, and which deepened more or less on contact with the air. In every case material in plain tin showed more loss of the original red, more browning, and greater purpling, in cases where purpling occurred, than in enameled tins.

## CHERRIES

A number of varieties of cherry, so chosen as to include both sweet and sour and to give a considerable range in the amount of pigment in the fruit, were used. These included three varieties of sweet cherries (*Prunus avium*), Baumann May (Guigne de Mai), Napoleon (Royal Ann), and Meeker; three varieties of sour cherries (*P. cerasus*), Early Richmond, Montmorency, and St. Medard, and one hybrid between *P. avium* and *P. cerasus*, Nouvelle Royale. Each of these was preserved in both plain and enameled cans and in glass. The fruit was exhausted 2 minutes and processed 45 minutes at 85° C.

## SWEET CHERRIES

Meeker is a light red fruit of very low acidity, Napoleon is a yellow-skinned variety having a red blush, low in acidity. Baumann May was decaying badly and consequently was picked while underripe; the fruit used was heavily blushed with red and was quite acid. The three varieties behaved precisely alike in that all trace of red was lost in processing in glass, the fruit becoming a clear bright yellow. The fruit in both enameled and plain tin was likewise a clear yellow when opened, but developed a pinkish purple tinge in the liquid after standing overnight. There was a moderate degree of corrosion in the plain tins after eight months' standing in storage in the laboratory.

## SOUR CHERRIES

Of the fruits of this type, Early Richmond was somewhat under-ripe; hence was lighter in color and higher in acidity than the others. Montmorency was a deeper red and less acid, St. Medard was a uniform dark red from skin to stone and was only mildly acid. In all three the fruit processed in glass lost somewhat in brightness of color through diffusion of the pigments into the water, but the color was a clear red proportional in intensity to that of the fresh fruit and there was no pink or violet tint. In enameled cans after eight months' storage there was more intense color in all varieties than in glass, but there was considerable purpling in St. Medard. In plain tin, the fruit of all the varieties showed pronounced fading after 8 months. The liquid was a faint pinkish red with decided milkiness in Montmorency and Early Richmond and purplish red in St. Medard. After standing overnight in porcelain dishes both liquid and fruit were decidedly purple in all cases, intensely so in St. Medard. The contrast between the fruit in plain and that in enameled cans was very striking. Very slight purpling occurred in Montmorency and Early Richmond from enameled cans, although there was considerable in St. Medard. Two cans of Montmorency, one in plain and one in enameled tin, which were sealed and processed without previous exhausting, showed considerably more discoloration than the corresponding exhausted cans, while the unexhausted plain tin can had greater milkiness than the exhausted plain tin.

The hybrid cherry, Nouvelle Royale, was not fully ripe; hence was lighter in color and more acid than is typical of this variety. Fruit in glass faded to a faint pink, that in plain tin became almost colorless. Enameled cans preserved much more of the color than did glass. There was a faint purpling in plain tin after standing overnight and an almost imperceptible deepening of the color in the material from enameled cans.

## MULBERRIES

Three strains of mulberries selected by the late Walter Van Fleet were employed. They differed very little in color, acidity, or physical character. Their behavior in canning was identical. The color in glass after eight months' storage was a deep, dark red, very little, if at all, different from the fresh fruit. In enameled tin the color was very well preserved but slight purpling was present. In plain

tin the color was a deep bluish purple in both fruit and liquid and the cans were heavily corroded. After overnight exposure to air there was very little change in the material from enameled cans, but that from plain tin had become intensely bluish purple. The effect of enameled cans in preserving the original color of the fruit was nowhere more strikingly evident than in the mulberry.

#### INDIAN STRAWBERRY

The Indian strawberry (*Duchesnea indica*) is bright scarlet in color, the pigment being confined to the surface of the fruit. Color is entirely lost during processing, both in glass and tin, the fruit becoming a dingy reddish brown, which does not change on opening and aeration.

#### PURPLE CABBAGE

In glass, the pigment of purple cabbage is largely extracted by the water, forming a beautiful, clear wine-red solution and leaving the tissue faintly red. In plain tin both cabbage and liquid were reddish purple, which became more intense on opening. After 17 months' storage material in glass had not changed materially, while that in tin had become light brown, about the color of ordinary cooked cabbage, and the entire inner surface of the can was deep purple to purplish black in color, due in part to sulphide blackening and in part to precipitation of the purple pigment.

#### BEETS

Considerable fading of color, but no purpling, occurs in beets both in glass and tin. No suggestion of purpling is evident on heating fresh beet with stannous chloride, but if sufficient alkali to neutralize be added and the material heated with stannous chloride, a bluish-purple color appears and is followed by the throwing down of a purplish-black precipitate.

#### CRANBERRY

In cranberries canned in water, the liquid and the fruit became a clear deep red. Fruit in enameled cans differed very little from that in glass, but there was a slight purpling on occasional berries and a faint purple tinge in the liquid. After standing over night there was slight deepening of the purple tint in both berries and liquid. In plain tin there was very marked shifting of color from red to purple when the cans were opened four days after processing, and the color began to deepen immediately upon exposure to air, becoming pronouncedly purple overnight. The broken berries browned considerably as a result of oxidation of tannin when the fruit was emptied into a dish, and as a consequence the color of the fruit after exposure to air was blackish purple, the surrounding liquid being dark purple.

#### SUMMARY OF RESULTS

The results of these experiments with various fruits show that the appearance of different shades of pink, violet, or purple during processing in tin cans, followed by intensification of the purple color upon exposure to air, occurs in a wide variety of fruits having red or

black-red anthocyan pigments present in skin or flesh. In no case does such discoloration occur in fruits known to be free of anthocyan, or in the colorless portions of anthocyan-containing fruits. In consequence, the appearance of some degree of alteration toward purple when the material is brought in contact with tin would appear to be a general and characteristic reaction of the anthocyan pigments, which will be encountered in canning or other manufacturing processes which involve contact of the material with tin.

When anthocyan-containing material which has been canned in plain tin is stored for six to nine months, the color upon opening is decidedly bleached as compared with like material opened a few days after canning. Material opened shortly after canning becomes strongly purple when exposed to the air for a few hours; that which has been canned for some months and which, consequently, has become bleached or faded, undergoes a return of the color toward that present immediately after processing, but this is accompanied by more intense purpling than occurs in material opened just after canning.

Kohman has stated that anthocyan are bleached with the tin of plain tin cans, but that the original color can be restored by exposure to the air (18). It must be said that the writers have found in this work that the color which develops on exposure to air is not the same as that originally present in the fresh fruit or that present in the material after processing in glass, but that there is a distinctly deeper color, due to the presence therein of more or less violet, converting the original clear red to purple. While in very acid fruits the alteration is less marked than in those of low acidity, it is usually sufficiently great to be observed when the container is opened, and can scarcely be overlooked after the material has remained a few hours in contact with the air.

#### CHEMISTRY OF THE ANTHOCYANS AS RELATED TO THEIR BEHAVIOR IN CANNING

In order to secure more information as to the mechanism of the discoloration, aqueous extracts of the various fruits employed in the experiments just described were prepared by boiling a quantity of the fruit with water, expressing and filtering the liquid, dividing into portions, and modifying the hydrogen-ion concentration by graduated additions of citric acid or sodium hydroxide. The degree of color change produced by treatment of the unaltered juice with stannous chloride, ferric chloride, and aluminum chloride could readily be compared with the effect of similar treatments upon the same extract after addition of acid or alkali.

#### THE GRAPE

The pigments of the grape have received considerable attention. Willstätter and Zollinger (39, 41) have examined varieties of *Vitis vinifera*, and Anderson (1, 2) and Anderson and Nabenhauer (3, 4) have studied varieties representing *V. labrusca*, hybrids of *labrusca* with *aestivalis* and *vulpina*, and a hybrid containing strains of *vinifera*, *aestivalis*, and *rupestris*. The results of these investigators show that the pigments present in these varieties, representing most of the species from which cultivated grapes have been derived, are

members of the delphinidin group, being either oenin (delphinidin dimethyl ether) or derivatives closely resembling oenin.

Of the seven varieties of grapes employed by the writers, the greenish yellow variety Uhland appears to contain no anthocyan pigment, as no violet or purple color developed when aqueous extracts were treated with stannous chloride, either with or without modification of the hydrogen-ion concentration. Extracts of the two red and four black varieties examined<sup>3</sup> differed only in the intensity of the color developed. Extracts of the red varieties became faintly purple, those of black varieties deeply purple. The color developed in partially neutralized extracts was in all cases much more intensely purple. In acidified extracts the change became less and less marked with increasing acidity. It is to be expected that members of the delphinidin group of the anthocyan will show the color reaction here described, wherever they may be found.

#### THE CHERRY

Keracyanin, the coloring matter of the sweet cherry, *Prunus avium*, has been studied in a preliminary way by Willstätter and Zollinger. Its properties in so far as known appear to identify it with the cyanidin group of anthocyan (40). It appears to be partially converted into colorless form by heat, whether in presence or absence of tin metal or salts.

Aqueous extracts of both sweet and sour varieties of cherries were tested with stannous chloride, aluminum chloride, and ferric chloride. The purpling with stannous chloride or aluminum chloride and the blackening with ferric chloride were very intense in the extracts of the deeply pigmented sweet varieties, slight to moderate in those of the sour varieties. That the color change in the sour varieties is inhibited by the acidity of the extract is shown by the intense purple developed after partial neutralization. Upon the gradual addition of acid, the purple color developed with tin or aluminum or the blackening produced by iron progressively lessened, and sufficient acid restored the original clear wine red. As in other cases, the alternate addition of acid and alkali, with the resulting shift of color, might be continued indefinitely.

#### THE RASPBERRY

The pigment of the raspberry has been assigned by Willstätter and Bolton (35), on the basis of qualitative reactions, to the cyanidin group. Their only statement with respect to it is, "Auch die Himbeere und die Frucht der Eberesche (Vogelbeere) enthält ein Cyanidinglucosid," no report of the tests being made. In its behavior with tin the pigment resembles those of other cyanin-containing fruits. Aqueous extracts of fresh raspberries, as well as juices from fruits preserved for one year by processing in glass, were examined. Addition of aluminum chloride produced no change in color. With stannous chloride there was slight purpling. On the addition of an alkali, the juice to which no metal had been added became greenish

<sup>3</sup> Uhland is a hybrid of *Vitis labrusca* with *V. riparia*. The two red varieties, Rogers No. 13 and Catawba, are *labrusca-vinifera* hybrids. Of the black varieties, Worden is pure *labrusca*, Eumelan a *labrusca-vinifera-aestivalis* hybrid, Cloeta a *lincecumii-rupestris-labrusca-vinifera* hybrid, while the parentage of the unidentified variety, which was employed by mistake for a named variety, is unknown.

black; th. to which aluminum chloride had been added became distinctly purple; that with stannous chloride very deeply purple. By adding the alkali drop by drop, the color in the presence of either metal undergoes a progressive change from red with a barely perceptible tinge of violet on the addition of the first drop to intense purple as the neutral point is reached. When acid is added the original color, whether in presence or absence of metal, returns. By alternately adding acid and alkali the shift of color may be produced at will. This behavior is characteristic of both cyanin and delphinidin pigments encountered in this work. It would appear from the writer's observations that in order to secure the characteristic color reactions with metals, the acidity of the solution must be somewhat less in the case of cyanins than is necessary with delphinidins.

#### THE CRANBERRY

The cranberry contains a cyanin pigment, idaein, which is a monogalactoside of cyanin (38). The juice of freshly cooked cranberries was found by the writers to give no change in color when stannous chloride or aluminum chloride was added. On bringing the juice nearly to neutrality either of these reagents produces a faint purpling followed by the deposition of a purple precipitate. Iron chloride produces little or no change in the freshly cooked juice, but on partial neutralization a brownish black color develops after a few minutes standing in air.

#### THE BEET

Betanin, the pigment of the red beet, has been isolated by Schudel (31), who found it to be unique among anthocyan pigments in that the glucoside contained nitrogen to the extent of 8.6 per cent. It consequently appears that it does not belong to any one of the three groups of anthocyan pigments thus far established. This is significant, since very little alteration in the quality of the color was observed in the canning tests. The pigment apparently undergoes partial destruction or conversion to colorless form on heating, as considerable decrease in intensity of color occurs both in glass and tin. It does not show any purplish discoloration with metallic tin or stannous chloride after heating and exposure to the air. If fresh beets are sliced and cooked in presence of granular tin, the color is identical with that of controls cooked in distilled water. If cooked with granular tin and a little acetic acid, there is pronounced fading of color, and purpling at the surface of the liquid accompanied by formation of an iridescent film occurs on 48 hours' standing. If stannous chloride is added to the cooked beets and the solution is then neutralized, purple color develops, and a purplish black precipitate is thrown down from a faintly purple solution. The behavior of the pigment of beet with tin differs markedly from that of the pigment of the sweet cherry and of the grape.

The anthocyanins of the other species included in this study have not been sufficiently studied with respect to their chemical composition to make it certain whether all of them are derivatives of pelargonidin, cyanidin, and delphinidin, singly or in mixture, or whether some of them are derivatives of yet other fundamental com-

pounds. Detailed chemical investigation will be required to determine the nature of the pigment or pigments present in each case, since qualitative tests upon the crude pigments do not warrant the drawing of conclusions as to their chemical affinities, as Onslow (28) and Perkin and Everest (29) have pointed out.

#### THE STRAWBERRY

The strawberry material examined included more than 200 selections and strains representing a large number of varieties, and was of very widely differing character with respect to degree of acidity and amount of pigmentation. There was, consequently, no uniformity in behavior of the material canned in tin, as has already been stated. Material of Portia, a rather deeply colored berry, was employed in the tests of aqueous extracts with metals. It showed the same general behavior with respect to aluminum chloride, stannous chloride, and ferric chloride as the sour cherries. Addition of alkali intensified the purple color; addition of acid resulted in return to the original red. Material of Howard, a variety which contains a relatively small amount of pigment, was also employed. This berry faded in processing, even in glass, to dull brownish red. Juice of such material gave inconclusive results with stannous chloride and aluminum chloride, only a very faint purpling occurring with the former and none with the latter. The addition of acid caused the disappearance of the purple produced by tin salts, but the solution remained a dull brownish red with no intensification of the red. It is known from the work of Willstätter and his pupils that many anthocyanins readily isomerize to colorless forms. It is also known that the presence of acids depresses or prevents the formation of the metallic compounds. In strawberries with a small amount of pigment and considerable acidity, isomerization, together with the browning of the tannins and the effects of the acid present, may be responsible for the difference in behavior from deeply pigmented forms, both in the can and in the presence of salts of tin and aluminum. The facts observed do not warrant the formation of an opinion as to the class of anthocyan pigments to which the color or colors of the strawberry belong.

#### OTHER MATERIALS

In the other materials employed the color developed in tin was pink, pinkish red, purplish red, or purple, and in all cases intensification of the purple hue occurred when the material was left exposed to the air. The degree of alteration of color was in many cases clearly dependent upon the amount of pigment originally present in the fruit, faintly colored fruits giving faint pinks which deepened slowly to purple, as in the case of viburnum berries or light-red sour cherries. In *Crataegus* berries, the pigment is confined to the epidermal layers and is small in amount, and it is not clear whether the very slight purpling observed is due to partial destruction or decolorization of the pigment or merely to its small amount.

In the plum, raspberry, blackberry, mulberry, and red cabbage, the degree of alteration of color developed appears to be determined by the amount of pigment, but the resulting color is in every case some shade of purple. It ranges from rose red in the cranberry to a nearly pure violet in the mulberry, exposure to the air resulting in all cases in the intensification of the purple hue. With stannous chloride and aluminum chloride the same changes in color as described for

grapes, peaches, and cherries were observed, and when acid and alkali were added the behavior was similar to that in these fruits.

It seems clear that pigments belonging to both the delphinidin and cyanidin groups show the same general behavior in tin, and their aqueous solutions show color reactions of the same general character when treated with tin and aluminum salts, namely, a shifting of color toward violet with alkali and a return to red with acid. Minor differences in behavior have been noted. It is possible that there are differences in the readiness with which the reaction takes place, depending upon whether a cyanidin or a delphinidin pigment is concerned. There may also be differences between members of the same group depending upon the state of the pigment, whether free or existing as mono- or di-glucoside. In so far as the pigments of the fruits here dealt with are known, nothing has been observed which can be considered as a specific reaction for any particular pigment, or for any one of the various forms of combination in which the pigment is known to exist. The pigment of the red beet is an exception, its behavior differing markedly from that of the other materials examined.

The behavior of the anthocyan pigments of fruits and vegetables when canned in tin is in complete agreement with the results of Shibata, Shibata, and Kasiwagi (32). The writers have found that the effect of heating anthocyan-containing materials in a container having a coating of metallic tin, or in glass in presence of metallic tin, tin salts, or aluminum salts, is to produce some degree of modification of the original color toward purple in the case of every material tested, with the exception of those cases in which the pigment is totally converted to colorless form under heat. If the effect of the combination of anthocyan with metals is to bring about a shift of color toward the violet end of the spectrum, regardless of the chemical constitution of the particular pigment concerned, the uniformity of behavior observed in a variety of fruits having pigments of unknown chemical structure is readily understood.

#### METHODS OF PREVENTING OR MINIMIZING THE ALTERATION OF ANTHOCYAN PIGMENTS IN CANNING

The difficulty encountered in securing satisfactory preservation of color in many materials when canned in plain tin has led to the introduction and extensive use of the inside enameled or lacquered can. George W. Cobb (9) states that this type of can, originally developed in Europe for the canning of certain meats and fish, was first employed for colored fruits in America in 1902 and that its use for red fruits and beets had become quite general by 1905. Bitting in 1912 (7) recommended its use for raspberries, cherries, plums, beets, pumpkin, and hominy. Such texts as those of Cruess (10), Zavalla (42), and Powell (30) recommend the use of the enameled can for acid or highly colored products. Merriman (24) has discussed the origin and chemical composition of various lacquering materials and has pointed out the limitations of their usefulness.

Concurrently with the introduction and adoption of the enameled can the canning industry has sought to continue the use of the less expensive plain tin can for some of the colored fruits by selecting for canning purposes the more deeply pigmented varieties or strains,

in the hope that a satisfactory degree of the original color might be retained when the material was preserved in plain tin. The results have been in part successful, in that a number of varieties of these fruits at present employed by canners can be put up in plain tin with a degree of preservation of color which makes them acceptable to consumers. In another respect the results have been disappointing. In the case of many fruits, the employment of the more heavily pigmented varieties of fruits results in more rapid generalized corrosion of the plain tin can or in more rapid pinholing of the enameled can than was encountered in the case of less deeply colored varieties of the same fruits. Attempts to increase the resistance of the can by modifications of the composition and methods of manufacture of the tin plate have been less fruitful than was hoped for (6, 12-15, 25-26). In consequence, the problem of corrosion and perforation is at present a more serious one than at any time in the history of the canning industry.

In the experiments here described, as well as in many others carried out in this laboratory in the past six or seven years, the anthocyan colors have in all cases been somewhat better preserved in enameled cans than in plain tin. In a few fruits, such as cranberries, the improvement due to the use of enameled containers, although distinct, is markedly less than in most materials. Even in the best enameled cans there is in all materials more loss of color than occurs in glass. Material canned in plain tin and opened a few days after processing shows considerable purpling and a milkiness of the liquid, neither of which is apparent in enameled cans of the same material opened after a like interval. When storage for six to nine months precedes the examination, considerable fading of the color is apparent in enameled cans, but is very much more pronounced in plain tin. On exposure to the air the material from plain tins undergoes marked intensification of color, finally approximating the purple tint observed on opening soon after processing. In enameled cans, some purpling occurs, but its amount is directly proportional to the area of metal exposed by imperfections in the lacquer.

#### ANTHOCYAN PIGMENTS IN RELATION TO CORROSION AND PERFORATION

The experience of canners with the various types of enameled cans and with plain tin as containers for the highly colored fruits and vegetables has led to recognition of the fact that the use of either involves difficulty. In the plain tin there is generalized corrosion of the inner surface with attendant loss of color on the part of the contents. In enameled cans there is better preservation of color but corrosion is localized at imperfections in the enamel, with the result that pinholing occurs in a relatively short time. In discussing this situation Baker (5) lists a number of fruits and vegetables which present especial difficulty. At the head of the list he places blackberries with strawberries next. The packing of these products he characterizes as gambling, not as a business. Rhubarb, blueberries, cranberries, black and red raspberries, loganberries, and blackberries are listed in the order of decreasing difficulty from the standpoint of perforation of cans. Baker recommends that those

which are sufficiently deeply colored to endure the resulting loss of color, such as blueberries, cranberries, and black raspberries, be placed in plain tin to prolong the period prior to perforation. Others which are not so heavily pigmented may be placed in enameled cans to preserve the color and considered as perishable goods by reason of the certainty that perforation will sooner or later occur. In making this recommendation of the use of plain tin for materials of this character Baker has left entirely out of account one very important consideration, namely, that the continued action of the material upon the surface of the plain tin may result in such an extensive absorption of tin by the contents that the wholesomeness of the material will become questionable.

In the experiments with the pigments of various fruits described in the preceding section, it became evident that the acidity of the solution was a very important factor in determining the amount of change in color produced by the addition of metal. In highly acid solutions there is little color change for the reason that the formation of the metallic salt of anthocyan is depressed or inhibited. It might be inferred from this observation that in highly colored fruits, those of higher acidity would exert less corrosive effect upon the metal of the can than those of low acidity. For the purpose of studying the action of some of the anthocyan-containing fruits upon the container in relation to such factors as acidity, tannin content, and oxygen content, experimental packs of all the fruits examined with respect to behavior of their pigments with metals, together with a number of the nonpigmented materials, were canned in both plain and enameled cans. A part of the material was exhausted prior to sealing, a part sealed without exhaust. The cans were then stored for a comparative study of the amount of corrosion. A part of the material was opened at the end of six months, a part at the end of nine months, and the remainder was held for observation as to perforation. The study of this material brings out a number of facts in regard to the factors affecting rate of corrosion in pigmented fruits.

A comparison of exhausted and unexhausted cans six to nine months after canning shows very clearly that oxygen content is an important factor in promoting corrosion. In every case, regardless of the pigment content or of the acid content of the material, the cans which had not been exhausted showed more extensive corrosion than did the exhausted controls. This is in agreement with the work of Kohman and others. The results indicate also that the presence of oxygen, as in unexhausted cans, may in certain instances lessen the production of free hydrogen. They indicate also that the presence of oxygen, together with anthocyan, greatly accelerates corrosion; that is, the effect of the two factors is additive.

The results with pigmented fruits show considerable evidence that corrosion of the can is materially decreased by the presence of high acidity in the material. The highly acid cranberry attacks the metal of the can much less extensively than do sweet cherries, blueberries, mulberries, strawberries, and black raspberries, all of which are much lower in acidity. The highly acid Oregon Evergreen blackberry does not attack the can as vigorously as does the black raspberry. Whether acid content is the sole factor responsible for the marked differences observed in these cases can not be stated defi-

nately, but certainly it is a factor. Oregon Evergreen blackberries and Logan blackberries combine high acidity and heavy pigmentation, but do not attack the cans to the extent that would be expected if the corrosive effect of these two factors were additive. In the cases just cited, and in a number of others encountered in this work, high acidity to a very considerable extent nullifies the corrosive action of the pigment, despite the fact that high acidity is itself a factor which increases corrosion. Rhubarb, for example, produces intense corrosion of the cans in which its acidity is an important factor. In those materials which are highly pigmented and at the same time highly acid, the acid depresses the formation of metallic salts of the pigment and thus decreases the rate and extent of the solution of metal. A considerable number of facts long on record in the literature without accompanying explanation become intelligible in the light of the experiments here described.

Studies carried out by Leach (23, p. 623-625) long since brought out the interesting fact that canned blueberries, having an acidity approximating one-twentieth normal, dissolved and carried into solution several times as much tin in a given time as strawberries or raspberries having twice the titratable acidity. Leach also tested various concentrations of malic, citric, and tartaric acids for their ability to dissolve tin from tin plate, finding that the pure tenth-normal acids dissolved only about one-tenth as much tin as did blueberries of one-half the acidity in equivalent time. The action of the pure acid ceased after about three months, no more tin being dissolved after nine months additional exposure. No such stoppage of action occurred in blueberries, which were nine times as efficient in dissolving the metal as canned tomatoes and twenty times as efficient as the pure acids. Leach could give no explanation of these facts, which are readily understood if we conceive of the anthocyan as continuously removing metal from combination with the acid and thus preventing the attainment of equilibrium between free acid and tin salt which occurs in pure acid solutions. It also explains results such as those of Donauer (11), who determined the rate of solution of metal of kettles used for Pasteurizing grape juice, concentrating apple juice, and cooking tomato pulp. The temperature for the grape juice was that of Pasteurization, while the other products were boiled down to an acid concentration somewhat exceeding that of the grape juice. The amounts of tin dissolved in equal periods of service bore the ratios, apple juice 80, tomato pulp 115, grape juice 380. The rôle of the anthocyan as a metal acceptor is here clear.

The reason why fruits of very low acid content but with large amounts of pigment, such as black cherries and blueberries, bring about more extensive corrosion of plain tin or earlier perforation of enameled tin than do more acid, less deeply colored fruits, such as red raspberries or sour cherries, is also clear. In the presence of large amounts of anthocyan, salts of tin with the acids of the fruits can have only momentary existence since they are immediately decomposed with transfer of the tin to combination with the anthocyan. In consequence there is sustained attack by the acid which may strip the tin completely from the steel plate, yet the amount of acid salts of tin present in the material will be very low. In fruits of

high acidity and low anthocyan content the initial attack upon the metal is vigorous but the anthocyan is presently exhausted by combination with the tin, after which equilibrium between free acid and metallic salts is reached, and further action upon the metal ceases. The limiting factor in corrosion is not acid content, but anthocyan content, since the capacity for taking tin into stable organic combinations determines the extent to which it may be attacked.

#### PERFORATION

The material stored for the study of perforation was held at room temperature. The cans were in all cases stored on end, with the end which had been sealed after filling turned up. In all the experimental material, perforation was practically wholly confined to enameled cans. The percentage of hydrogen swells occurring in the enameled cans was also very much greater than in plain tin. In every case, enameled cans which ultimately showed perforation first became hydrogen swells. In only two cases did side perforations occur, in all others the perforation was located either in the counter-sink or at the juncture of the side seam with the end. In counter-sink perforations the opening is not at the bottom of the groove, but at the top, usually on the side next the center of the can. This is the region of maximum strain as a result of expansion during processing. The juncture of side seam and end seam is a region which is subjected to mechanical stress in the making of the cans, as well as to abrasion of the enamel covering. The location of perforations is therefore in accordance with expectation based on a knowledge of the behavior of metal previously subjected to strain when exposed to conditions favoring corrosion (33).

Packs of nonpigmented material prepared for comparison with pigmented material in the perforation studies gave no results since perforation failed to appear in such material.

It is apparent from the results of the author's comparative studies of the anthocyan-containing fruits that the amount of purple discoloration produced by contact with metal is a rather dependable indication of the ability of the material to perforate enameled cans. This holds true for the fruits of a considerable number of species which have been examined, and also for varieties and strains within a single species. Some 200 horticultural varieties and strains of strawberry have yielded evidence on this point. The most heavily pigmented varieties or strains were the first to perforate. Such deeply colored varieties as Portia and Progressive began to develop perforations after about six months, long before perforation began to occur in less highly pigmented varieties, such as Howard 17. The varieties of strawberries employed varied widely in acidity and in tannin content as well as in anthocyan content. The independent variations of acidity and tannin content complicate the results of the perforation study. Any substance which can take the dissolved metal out of combination with the acids of the fruit, thus setting the acid free to continue action upon the metal, will favor perforation or corrosion. Tannins and other substances capable of combining with the metal may function as aids to these processes just as anthocyanins do. Consequently perforation, like corrosion, is favored by a variety of causes, of which the presence of anthocyan is only one.

The problem of discoloration is directly related only to such corrosion as is specifically due to the presence of anthocyanins.

Kohman has given special attention to the problem of perforation by fruits containing anthocyanins (17-22). He has shown that acidity of the fruit is not the most important factor determining perforation, since different products of like acidity but unlike pigment content show very large differences in the readiness with which they produce perforation. Kohman's results have led him to the belief that the rôle of anthocyan in perforation is that of a hydrogen acceptor—that is, the pigment takes up the hydrogen produced, so that perforation occurs without previous perceptible swelling of the can. The results obtained by the present writers indicate that anthocyan also functions as a metal acceptor, increasing the capacity of the material to dissolve metal by taking it into stable organic combination as rapidly as it is dissolved.

#### SUMMARY

Violet, pink, or purple discolorations in fruits and vegetables canned in tin are of rather widespread occurrence. In some materials such discolorations occur regularly, being accepted as a matter of course; in others their appearance is occasional and sporadic, resulting in considerable losses by causing the material to be discarded as unfit for food. Such discoloration has received various explanations, none of which are more than partially correct.

Discolorations of this type occur only in such fruits and vegetables as contain anthocyan pigments in skin or flesh, and are confined to the pigmented portions of the material. If pigmented and nonpigmented portions are separated prior to canning, discoloration appears in the pigmented portion but is absent from the nonpigmented portions.

The discoloration occurs only in material canned in tin, but occurs in both plain and enameled cans. When processing is carried out in glass, the original color of the material is preserved except for lessening in intensity due to partial conversion into colorless form by heat. In tin cans, a greater loss of color occurs, accompanied by conversion of some of the pigment to violet and consequent shifting of the color of the product toward purple. This alteration in color is intensified by opening the cans and exposing the material to the air.

The amount of anthocyan pigment developed in such fruits as have the pigmentation confined largely or wholly to the skin, as the red-skinned peaches, is determined in considerable degree by seasonal conditions. High temperatures and intense sunlight result in development of unusually high color in such fruits, and this results in the appearance of discoloration in the canned product.

The appearance of discoloration is due to the reaction of the anthocyan with tin dissolved from the container, and results in the formation of complex metallic compounds of anthocyan which are violet in color.

The anthocyan of the peach has been isolated and partially purified. Its behavior toward metals is similar to that of other anthocyan pigments.

The behavior of a large number of anthocyan-containing fruits and a few vegetables, including peaches, plums, sweet and sour cherries, apples, red and black raspberries, blackberries, currants,

grapes, mulberries, berries of *Viburnum* and *Crataegus*, red cabbage, and beets, has been studied by means of canning tests in plain and enameled tin and in glass; as well as the comparative behavior of aqueous solutions of the pigments with metals.

The formation of violet-colored salts with tin or tin salts is a general property of the red anthocyan pigments. It occurs when the anthocyan-containing material is heated in contact with tin, as in canning, when aqueous solutions of the crude or partially purified pigment are heated with metallic tin or aluminum, or when salts of these metals are added in the cold. The amount of the violet compound formed is determined by the amount of the pigment present, and by the degree of acidity of the medium, low acidity favoring its formation, high acidity depressing or suppressing it.

The color changes produced by combination with metals are reversible. Addition of alkali to a solution of pigment containing a metal results in formation of more salt with resulting intensification of violet color; addition of acid destroys the combination and restores the original red color.

In anthocyan-containing canned material the metal salts formed by combination of the acids of the fruit with the can metal are broken up by transfer of the metal into combination with the pigment, releasing the acid to continue attack upon the can. If the initial acidity is low, thereby favoring formation of the metal salt, extensive attack upon the can will occur, continuing up to the limit of the metal-absorbing capacity of the pigment. If the acidity is high, the formation of the metal-anthocyan compound is thereby reduced or suppressed, solution of the metal is limited to the absorbing capacity of the acids present, and equilibrium is reached before extensive corrosion occurs.

The enameled can preserves the original color of red fruits and reduces discoloration by decreasing the contact between pigment and metal. At the same time, it increases the rapidity with which perforations of the metal occur by limiting the area from which metal can be removed.

The processes resulting in discoloration are intimately related to the problem of corrosion and perforation, although these involve other factors. Anthocyan plays a very important rôle in causation of corrosion and perforation wherever it is present, but these processes can also be brought about by other compounds than anthocyan.

The principal factors concerned in corrosion are oxygen, acidity, anthocyan, and tannin. Some of these factors stand in antagonistic relationship. High acidity generally favors corrosion but depresses the formation of metal-anthocyan compounds and may thus retard corrosion. High acidity represses oxidation of tannin and formation of hydrous stannous oxide. Oxygen accelerates corrosion and increases the total activity by oxidizing the ferrous and stannous salts to the corresponding ferric and stannic states. The interrelationships of the factors concerned are so complex that practically every substance canned presents a specific problem.

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# WHY APPLICATIONS OF NITROGEN TO LAND MAY CAUSE EITHER INCREASE OR DECREASE IN THE PROTEIN CONTENT OF WHEAT<sup>1</sup>

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## INTRODUCTION

That low-protein wheat is due to the insufficiency of supply of nitrogen available to the plants during the later-growth stages has been shown by several investigators (1, 2, 4).<sup>2</sup> Thus it has been found that the application of soluble nitrogen to the plants at various stages of development up to a certain growth phase resulted in wheats that varied correspondingly in the protein content of the grain. The later nitrogen was applied, the higher was the protein content of the grain. However, as the literature also contains a fairly large number of references to investigations (3, 5, 6, 7) wherein the addition of nitrogen to land either failed to improve the protein content of wheat or actually caused a marked decrease in the percentage of this constituent, it is the purpose of this paper to set forth the conditions under which such results may be obtained.

Results obtained by the writer from investigations in which one of each of the essential salt elements was absent from the culture media of wheat for various periods of time following the initial exposure of the cultures to complete nutrient solutions, show that the absence of either of the two elements calcium and nitrogen, profoundly affect (decrease) the protein content of the grain. The percentage of protein in the grain obtained from cultures grown ten weeks in a complete nutrient solution and then transferred to media devoid of calcium was about one-half that obtained from cultures not deprived of any essential salt element; and in similar series in which nitrogen was omitted from the media, the grain contained about two-thirds the normal percentage of protein. Alkaline or neutral reactions of the media during the later growth phases of the plants caused a slight decrease in percentage of protein. The absence of potassium, phosphorus, or sulphur in the culture media after the plants had grown four weeks in complete culture solutions had no effect on the protein content of the grain. Similar experiments with magnesium showed that this element can be dispensed with after the plants have grown four weeks in complete culture media, with only slight decrease in protein. Thus it appears that variation in the protein of grain is primarily related to the nitrogen and calcium relations of the growth media.

## FACTORS CAUSING VARIATION IN PROTEIN CONTENT OF GRAIN

The percentage of nitrogen or any other constituent in plant tissue is obviously a factorial measurement of the quantity of element that the plant has absorbed and of the growth it has made as the result

<sup>1</sup> Received for publication Feb. 23, 1927; issued August, 1927.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 139.

and in part as an incidence of absorbing that quantity of the element. That the measure of growth (stated in terms of mass) plants make from any given quantity of nitrogen absorbed may vary markedly is only another way of stating that wheat varies in its percentage of this constituent. Thus, the problem of accounting for variation in the protein content of the grain resolves itself into a consideration of the factors which determine the mass of grain material that will be produced and into which a given quantity of nitrogen will be distributed and assimilated as protein.

Obviously nitrogen is required by wheat for the production of non-grain tissues as well as grain tissue. Furthermore, the quantity of grain tissue formed by a plant bears certain relations of proportionality to the quantity of nongrain tissue formed. The factors that bear on the apportionment of nitrogen to these two classes of tissue conceivably must be important in a consideration of the cause that effected the marked differences above referred to, in the responses from applications of nitrogen to land. In one set of cases fertilization with nitrogen gave rise to high protein grain and in another set to low protein grain.

#### DESCRIPTION AND RESULTS OF EXPERIMENTS

Marked differences were brought out very clearly by the writer in an investigation in which an array of varieties of spring wheats were treated with applications of sodium nitrate at various stages of their development. Furthermore, relations developed among the varieties that have made it possible to explain the paradoxical results of different investigators.

Briefly described, the experiments were as follows: Stone jars were filled with a fairly good soil and these were seeded with nine varieties of wheat. Each variety was treated separately, but all varieties were treated alike so that comparison could be made as to the effects of applications of nitrogen to different varieties at different stages of growth. The salt was supplied in solution upon the surface of the soil at a rate equal to an application of 100 pounds of nitrogen per acre. Soon after the seedlings appeared above the ground they were thinned to seven plants per jar. Mention is made of this at this time, as it will be shown later in this paper that the number of stalks produced by tillering had a very pronounced effect on the apportionment of nitrogen for grain and nongrain tissue. Table 1 summarizes the results of experiments made to compare the effect produced on the protein content of grain by nitrogen applied to different varieties of wheat at different stages of growth.

Several features of Table 1 need mention. It is to be noted that the percentage of protein in the grain of cultures which received nitrogen 30 or 90 days after planting was in every case greater than that obtained in grain from untreated cultures. With one exception, cultures that received nitrogen 90 days after planting produced grain higher in protein than did the cultures that received nitrogen at an earlier growth period. The exception referred to was noted in the case of Marquis.<sup>3</sup> The culture of this variety that received nitrogen 90 days after planting produced grain perceptibly lower in protein than did the cultures that received nitrogen 30 days after planting.

<sup>3</sup> This feature can be explained by a certain characteristic of the plant, namely, by the degree any treatment delays the maturity of the plants, the longer the delay the more remote is the application of nitrogen to the later growth period when it can fertilize nitrogen more efficiently for protein in the grain.

However, in either case the protein content was decidedly higher than that of the grain from untreated cultures. Disregarding this one exception, it may be stated that the markedly higher values obtained for the cultures that received nitrogen 90 days after planting, as compared with those for the cultures supplied with nitrogen at earlier growth periods is in general accord with the principle that the efficiency with which wheat plants utilize nitrogen for protein in grain is related to the proximity to the period of grain formation at which a given supply is absorbed by the plants.

TABLE 1—*Percentage of protein in grain of cultures that received nitrogen at different growth stages*

Treatment	Varieties of wheat								
	Bunyip	Cedar	Dart's Imperial ✓	Early Baart	Fulcaster ✓	Hard Federation ✓	Marquis	Sonora ✓	White Australian
No nitrogen applied.....	Per cent 8.94	Per cent 9.51	Per cent 8.28	Per cent 8.76	Per cent 8.34	Per cent 12.21	Per cent 8.50	Per cent 7.88	Per cent 9.0
Nitrogen applied at time of planting.....	9.60	10.67	7.35	8.84	7.32	9.79	8.92	7.15	9.0
Nitrogen applied 30 days after planting.....	13.62	12.14	10.76	10.98	9.33	12.05	11.60	9.81	9.2
Nitrogen applied 90 days after planting.....	15.54	15.55	11.88	13.03	11.90	14.76	11.00	11.98	12.1

The most interesting feature of Table 1, however, is the comparison it affords of the values for protein of the cultures that did not receive nitrogen and those that did receive it at the time of planting. This comparison shows that the values for protein of four varieties—Dart's Imperial, Fulcaster, Hard Federation, and Sonora—were less in the grain of the treated than in the grain of the untreated cultures. The values for White Australian in treated and untreated cultures were equal. Thus, in five of the nine varieties, application of nitrogen to the soil at the time of planting either had no effect or was more or less harmful to quality in wheat, as defined by the protein content of the grain. Table 1 shows that applications of nitrogen to plants at certain growth periods results in the production of high-protein grain, and likewise that applications of nitrogen at other times has a harmful effect. To explain these varying results it is necessary to consider the effect a given growth phase has upon the apportionment of nitrogen to the two classes of tissue, grain and nongrain.

Table 2 shows the relative increase in tillering of nine varieties of spring wheat resulting from application of nitrogen at different growth periods.

Table 2 gives important information concerning growth characteristics of different wheat varieties. It shows (what is well known) that varieties possess markedly different properties in respect to tillering. Differences were obtained in tillering within given varieties, as well as among varieties. The outstanding features, however, can be briefly stated as follows:

(1) All varieties except Dart's Imperial showed good tillering from nitrogen treatment.

(2) Dart's Imperial tillered very sparsely, regardless of the treatment employed. This wheat, because of genetic reasons, is considered incapable of tillering profusely.

(3) Bunyip, Cedar, Early Baart, Marquis, and White Australian, gave maximum tillering when nitrogen was applied 30 days to 90 days after planting. They tillered very sparsely when nitrogen was applied at the time of planting. Several characteristics of these varieties, however, need further mention, namely: They possess the ability to tiller, which is a heritable character of these varieties; their ability to tiller is conditioned on certain growth phases; tillering is dependent on an ample supply of available nitrogen.

(4) Fulcaster, Hard Federation, and Sonora tillered profusely with the first treatment, as well as with the later treatments. These varieties, therefore, differed from the ones mentioned above in that the growth phase did not condition the tillering properties of the plants.

It may be said, therefore, that tillering expresses both genetic and physiological properties of plants. As has been shown, however, variation in the protein content of grain of any given variety of wheat is due to physiological processes. If, therefore, tillering affects the protein content of the grain, it may be assumed to have some influence on the processes that affect the supply and apportionment of nitrogen to the grain and nongrain tissue.

TABLE 2.—*Relative increase in tillering of nine varieties of spring wheat due to application of nitrogen at different growth periods*  
(Untreated cultures considered as basis, namely, 100)

	Bunyip	Cedar	Dart's Imperial	Early Baart	Fulcaster	Hard Federation	Marquis	Sonora	White Australian
Period after planting at which nitrogen was applied (days):									
0.....	104	133	102	109	126	140	117	204	116
30.....	171	212	108	214	129	156	218	188	140
60.....	194	224	127	267	150	198	197	219	156
90.....	275	259	115	267	160	258	197	213	186
Coefficient of tillering capacity *	2.64	1.95	1.13	2.45	1.27	1.84	1.68	1.04	1.60

\* Obtained by dividing the highest value by the lowest value for each variety.

Tillering is essentially a process of vegetative development. Vegetative development precedes grain formation. If the supply of available nitrogen in the soil is less than the amount the plants can absorb and utilize, therefore it follows that excessive development of vegetative growth with incident demand on the limited supply of nitrogen accentuates the dearth of nitrogen during the later period of growth. Some of the nitrogen required for vegetative production can not be utilized later for protein in the grain. A certain minimum amount becomes a part of the nongrain tissue. The more there is of this tissue, the less is the quantity of nitrogen available for grain. But the amount of nongrain tissue that may be formed by a plant is limited by the length of the growth period. A given quantity and state of vegetative development precedes grain formation. Varieties like Fulcaster, Hard Federation, and Sonora that begin to tiller at very early growth stages have a relatively longer period in which to produce nongrain tissue than have varieties that tiller relatively late. Such varieties consequently can use relatively larger quotas of nitrogen for straw and leaves. If the supply is limited, obviously this would leave less nitrogen in the soil to be absorbed later for grain than in the case of those varieties that tiller relatively late in their life cycle.

Table 2 further shows that certain differences in the tillering properties of varieties have a definite relation to the variations in the percentage of protein resulting from the application of nitrogen at different periods of plant growth. Under the caption "Coefficient of tillering capacity of the varieties" is given the relative increases in tillering resulting from the application of nitrogen at a propitious growth period over that which was least propitious. It is to be noted that the lowest values resulted for Dart's Imperial, Fulcaster, and Sonora. These are three of the four varieties the untreated cultures of which produced grain higher in protein than did cultures treated at the time of planting.

The coefficient of tillering capacity as here applied is the quotient obtained by dividing the maximum tillering by the minimum. Low values may result (1) from varieties that do not tiller or that do it very sparsely at any growth phase (2) and from varieties that tiller equally abundantly at all growth stages. Both of these classes were represented among varieties that gave low values.

In Table 3, the data on tillering are analyzed further. The tillering percentage obtained from the cultures that received nitrogen 30 days after planting is divided by that of the cultures that received nitrogen at the time of planting. The values obtained give the tillering capacity of the varieties as a property of their early growth phase only.

TABLE 3.—*Increase in tillering of varieties of wheat resulting from the application of nitrogen 30 days after planting as compared to that obtained from the application of nitrogen at the time of planting*

Variety	Tillering increase from nitrogen application 30 days after planting	Variety	Tillering increase from nitrogen application 30 days after planting
	<i>Per cent</i>		<i>Per cent</i>
Bunyip.....	164	Hard Federation.....	111
Cedar.....	159	Marquis.....	186
Dart's Imperial.....	104	Sonora.....	92
Early Baart.....	196	White Australian.....	121
Fulcaster.....	103		

The four lowest values are those for Dart's Imperial, Fulcaster, Hard Federation, and Sonora. These are the four varieties that produced higher percentage of protein in the grain from untreated than from treated cultures. It should be noted that White Australian stands next in order to the four varieties above mentioned in percentage of protein produced in the grain. The percentage of protein in the treated and untreated cultures of this variety was equal.

As already indicated, the low tillering capacity values of the varieties may arise from one of two causes—(1) inability of the variety to tiller or (2) ability to tiller profusely at all growth stages. The relation between the low values of increase in tillering capacity and the value of protein in the grain resulting from the application of nitrogen 30 days after planting, brings up the question as to how these factors are related. It will be recalled that Dart's Imperial is representative of the first class, that did not tiller because of genetic

reasons. However, the plants of this variety did have the ability to absorb and utilize nitrogen equally well at all growth stages. Tillers were not necessary in plants of this variety to provide an avenue of increased vegetative growth as the result of adding nitrogen to the soil. The parent stalk at any growth stage had the capacity to expand sufficiently without tillering to take care of the increase in vegetative production that resulted from increased absorption of nitrogen. This is attested by the thick, heavy stalks this variety produced. Thus, the ability of Dart's Imperial to absorb relatively large quotas of nitrogen when supplied at the time of planting resulted in two reactions that precluded the production of high protein grain. In the first place the vegetative growth gave inception to too much grain for the nitrogen subsequently available; and in the second place nitrogen became deficient in the later-growth stage because the supply at the beginning of the season was insufficient to withstand the draft occasioned by the degree of growth this variety could make.

The Sonora variety is a representative of the second class of wheat which possesses the property to tiller equally abundantly at any growth stage, provided sufficient nitrogen (and, obviously, the other necessary elements) are available. Sonora could not expand sufficiently in the parent stalks to provide for all the vegetative growth resulting from the absorption of nitrogen during the early growth stages. Its tillers, however, developed very early. They had a longer growing period than did tillers that arose later; consequently they made a relatively greater draft on the supply of nitrogen in the soil than did the plants that tillered later. Capacity to absorb nitrogen may be considered as related factorially to the length of the growing period and to the mass of vegetative tissue produced. The relatively large vegetative development gave inception to more grain than would have resulted from a lesser vegetative development. Since the supply of nitrogen was insufficient for this increased quantity of grain, this grain was as a result lower in protein content than would have been the case had the yield been lower.

Inasmuch as the reason why the varieties Sonora and Dart's Imperial produced a higher protein grain from the untreated cultures than those to which nitrogen was applied at the time of planting is the effect which the early growth period has upon the subsequent supply of nitrogen, it is evident that both the soil factor and the plant factor are involved. It is clear, therefore, that by manipulation of either of two factors—i. e., by supplying nitrogen to the soil during the later growth period of the plant or by curtailing excessive grain production—it is possible to produce high-protein grain even under any condition.

Between Dart's Imperial and Sonora, representative of two types of wheats having distinctive genetic characters as to tillering, stand Fulcaster, Hard Federation, and White Australian. The growth habits of Fulcaster in respect to the features here discussed are more nearly akin to those of Dart's Imperial than Sonora. In respect to its ability to set tillers from applications of nitrogen at the time of planting, Hard Federation is similar to Sonora. However, it differs markedly from Sonora in that it has the property to increase in tillering capacity with growth. White Australian is a variety on the border line in respect to the effect which applications of nitrogen at the early growth stage have upon the protein content of the grain.

The treated and untreated cultures produced grain having the same protein values. Inspection of the data shows that the growth habits of this variety resemble those of Bunyip, Cedar, Early Baart, and Marquis more than they do the habits of the varieties in which applications of nitrogen at the time of planting produced lower protein values than were obtained for the untreated cultures.

### CONCLUSIONS

It may be stated, therefore, that the supply of nitrogen available for the quantity of grain that any state of vegetative development may induce determines the protein content of any sample of wheat. It has been shown in this paper that the amount of nitrogen available to the plants at different growth stages affects the protein content of the grain. It appears, therefore, that some of the contradictory results obtained and reported in this field covering the effect of fertilizer applications on the protein content of wheat, may be explained by the peculiarities of the varieties. It seems well to emphasize here, also, a point that has not received the consideration in fertilizer experimentations that its importance merits, namely, that the properties of wheat varieties can and do markedly affect the efficiency of any fertilizer treatment.

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# METABOLISM OF NITROGEN COMPOUNDS IN DORMANT AND NONDORMANT POTATO TUBERS<sup>1</sup>

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## INTRODUCTION

In a previous paper by the present writer<sup>2</sup> it was shown that nitrates stimulate growth in dormant potato tubers to a greater degree than in nondormant tubers. The investigation herein reported indicates that the stimulation of growth by nitrates may not be due to an internal deficiency of soluble nitrogen compounds in dormant tubers that can be offset by the absorption of nitrate, as was suggested in the earlier paper.

## EXPERIMENTAL DATA

### INFLUENCE OF STORAGE TEMPERATURES AND NUTRIENT MEDIA ON LENGTH OF DORMANT PERIOD

One half of a uniform lot of immature tubers were stored at 5° C., the other half at room temperature (approximately 20°), and at intervals of two weeks representative samples from each group were planted in pure quartz sand. These quartz-sand cultures were divided into three groups. Two of the groups were supplied with complete nutrient solutions. The nitrogen in one occurred in the form of nitrate and in the other as an ammonium salt. The first solution contained the following salts in equal molal concentrations (0.007), KNO<sub>3</sub>, MgHPO<sub>4</sub>, and CaSO<sub>4</sub>; the second contained equal molal concentrations (0.005) of KH<sub>2</sub>PO<sub>4</sub>, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, MgHPO<sub>4</sub>, and CaSO<sub>4</sub>. The third group was supplied with a nitrogen-free solution similar to the first in composition and concentration except that KNO<sub>3</sub> was replaced by KH<sub>2</sub>PO<sub>4</sub>. Confirming the results obtained in previous experiments by the writer, Table 1 shows that the presence of nitrate tends to abbreviate the dormant period, but that the use of ammonium salts does not affect it; for there is no significant difference in the time of sprouting between the ammonium and the nitrogen-free cultures.

### METABOLISM OF NITROGEN COMPOUNDS DURING THE DORMANT PERIOD

At intervals of two weeks during the dormant period samples of tubers were rapidly frozen, pulverized, and the juice expressed under approximately constant pressure (12.5 kgm. per square centimeter). The pulp residue was then extracted with distilled water and dried. The total nitrogen of this pulp residue progressively decreased, a total decrease of approximately 30 per cent occurring during the 10-week storage period at room temperature and approximately 20 per cent at 5° C. When the tubers were stored at room temperature

<sup>1</sup> Received for publication Mar. 22, 1927; issued August, 1927.

<sup>2</sup> NEWTON, W. THE REST PERIOD OF SOLANUM TUBerosUM IN RELATION TO AVAILABLE NITROGEN. *Science* (n. s.) 58: 207-208. 1923.

the amino nitrogen in the expressed juice, as determined by the standard Van Slyke method, increased slightly with each successive analysis. The total increase during the 10-week storage period was from 15.4 to 17.5 mgm. of nitrogen per 10 c. c. of juice. When the tubers were stored at 5° the amino acid decreased slightly during the first four weeks, but this decrease was followed by an increase. However, at the end of the 10-week period the amino-acid content of the juice was not markedly greater than at the beginning. There was a significant progressive increase of amide nitrogen<sup>3</sup> in the expressed juice from the tubers stored at both temperatures; an increase during the 10-week period of from 4.9 to 7.2 mgm. per 10 c. c. of juice when the tubers were stored at room temperature, and from 4.9 to 6.7 mgm. when stored at 5°. Although the actual concentration of amino and amide nitrogen was greater in the expressed juice when the tubers were stored at room temperature, Table 1 indicates that the tubers stored at 5° tend to sprout first.

TABLE 1.—Time from planting to sprouting of potato tubers treated with complete nutrient solutions and with nitrogen-free solution

Number of weeks in storage	Temperature at which stored (° C.)	Date of planting	Average number of days from planting to sprouting (12 cultures)		
			Complete nutrient-solution cultures		Nitrogen-free cultures
			Nitrate	Ammonium	
2	5	Apr. 20	38	63	61
2	20	do	42	63	61
4	5	May 4	38	46	47
4	20	do	40	50	48
6	5	May 18	27	40	39
6	20	do	33	45	42
8	5	June 1	13	30	30
8	20	do	19	35	36
10	5	June 15	7	21	20
10	20	do	15	24	25

#### METABOLISM OF NITROGEN COMPOUNDS AS INFLUENCED BY STIMULI

The fact has long been recognized that stimuli which increase the rate of respiration in potato tissue will abbreviate the dormant period. Following the methods of Appleman<sup>4</sup> and Müller-Thurgau<sup>5</sup> the writer found that respiration was stimulated by (1) removing the skins; (2) treating the tubers with hydrogen peroxide; (3) storing at 0° C.; and (4) incubating at 35°. The expressed juice from the tubers thus treated contained 10, 12, 13, and 2 per cent more amino acid than the juice from tubers of the original stock. Since an error of 2 per cent was involved in the analytical procedure the last value may not be significant. In every case there was an increase of amide nitrogen as a result of these treatments; an increase of 10, 14, 36, and 8 per cent, respectively.

<sup>3</sup> The NH<sub>3</sub> evolved under slightly alkaline conditions after hydrolyzing for two hours with 4 per cent HCl at 100° C.

<sup>4</sup> APPLEMAN, C. O. BIOCHEMICAL AND PHYSIOLOGICAL STUDY OF THE REST PERIOD IN THE TUBERS OF SOLANUM TUBEROSUM. Md. Agr. Expt. Sta. Bul. 183, p. 181-226, illus. 1914.

<sup>5</sup> MÜLLER, H., Thurgau, and SCHNEIDER-ORELLI, O. BEITRÄGE ZUR KENNTNIS DER LEBENS-VORGÄNGE IN RUHENDEN PFLANZENTEILEN, I-II. Flora (n. F. 1) 101: 309-372, illus., 1910, (n. F. 4) 104: 387-446, illus., 1912.

## PROTEOLYTIC ENZYME ACTIVITY IN POTATO JUICE

Proteolytic enzyme activity was more intense in the expressed juice of nondormant than in that of dormant tubers, as indicated by the accumulation of amino acids upon incubating the juice, under toluol at 30° C. For example, at harvest, and at four and eight weeks later, the increase of amino acid was 0.1, 0.9, and 2.3 mgm., respectively, in 10 c. c. of juice during a 48-hour incubation period. Results of this type were obtained only when the incubation period was short, for the initial increase of amino-acid nitrogen in the juice was invariably followed by a decrease. In one sample of juice the amino-acid nitrogen increased from 12.6 to 14.6 mgm. per 10 c. c. over a 24-hour incubation period at 40°, but over a 100-hour period the same juice contained only 13.5 mgm. This phenomenon, an increase followed by a decrease, was typical of the results obtained with all the samples of juice studied. The data from one sample of nondormant tuber juice was used to construct curve 2, Figure 1, to illustrate this general phenomenon.

The disappearance of the amino-acid nitrogen may be accounted for by the enzymatic conversion

of amino to amide nitrogen, for the amide nitrogen increased with the time of incubation and showed no maxima followed by a decrease. For example, during a 24 and a 100 hour incubation period of 40° C. the amide nitrogen in one sample of juice increased from 3.8 to 4.0 mgm. and from 3.8 to 4.3 mgm., respectively, in 10 c. c. of juice. Similar results were obtained with all the samples of juice studied. The data from one sample of nondormant tuber juice

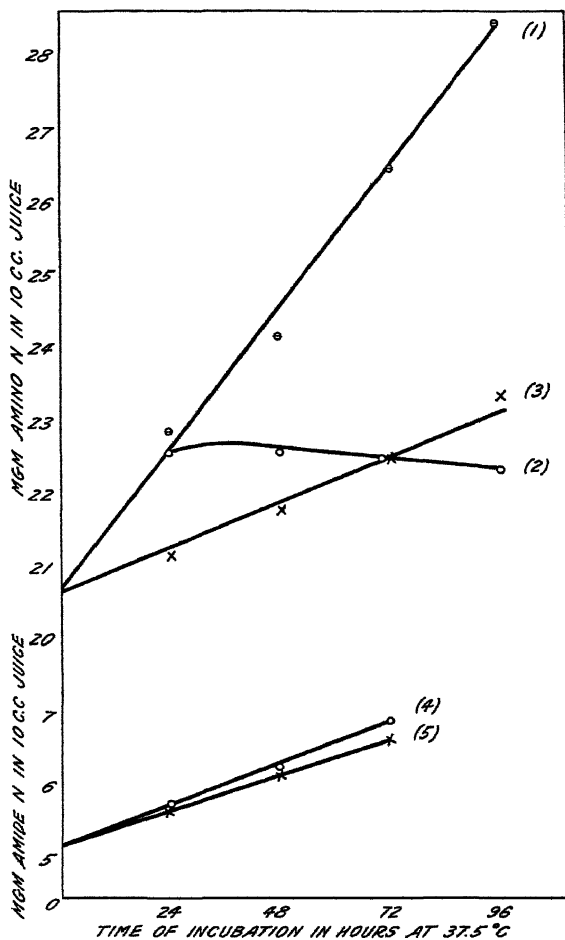


FIG. 1.—The influence of the addition of casein and calcium nitrate to nondormant potato juice incubated under 2 per cent toluol: Curve 1, juice containing 2 per cent casein; curve 2, juice alone; curve 3, juice containing 1 per cent  $\text{Ca}(\text{NO}_3)_2$ ; curve 4, juice alone; curve 5, juice containing 1 per cent  $\text{Ca}(\text{NO}_3)_2$ .

was used to construct curve 4, Figure 1, to illustrate this general phenomenon.

Proteolytic enzyme activity may be demonstrated with greater ease by adding casein to the potato juice before incubating, for, unlike the results with the juice alone, after the addition of casein there was an accumulation of amino acids over a long period as approximately a straight-line function of the time of incubation. The data from one sample of nondormant tuber juice containing casein was used to construct curve 1, Figure 1, to illustrate the general nature of the results obtained.

When small quantities of calcium nitrate were added to the potato juice the rate of increase of both amino (fig. 1, curve 3) and amide nitrogen (fig. 1, curve 5) was inhibited. The inhibition was not so pronounced, however, in the case of amide nitrogen.

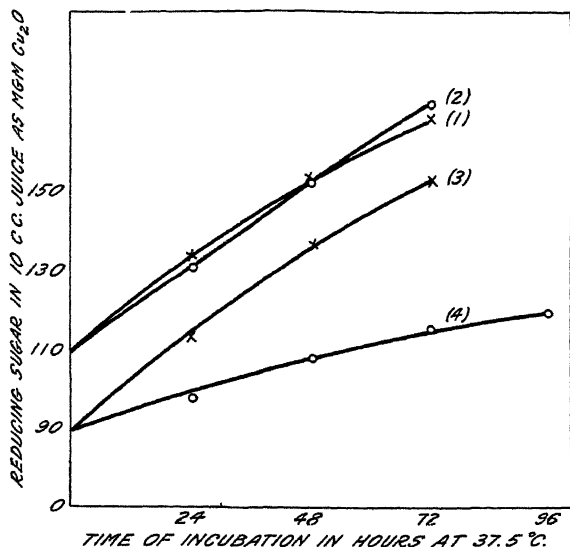


Fig. 2.—The influence of the addition of asparagine to nondormant and dormant potato juice incubated under 2 per cent toluol: Curve 1, nondormant juice containing 1 per cent asparagine; curve 2, nondormant juice alone; curve 3, dormant juice containing 1 per cent asparagine; curve 4, dormant juice alone

of dormant tubers. The addition of asparagine to dormant tuber juice increased the rate of accumulation of reducing sugar (compare curves 3 and 4, fig. 2), but had no significant effect upon nondormant tuber juice (fig. 2, curves 1 and 2). The reducing sugar was determined by the copper gravimetric method as modified by Davis and Daish.<sup>6</sup>

#### DISCUSSION

The stimulation of growth by nitrates may be due to the rule observed by Appleman,<sup>7</sup> namely, that oxidizing agents tend to stimulate growth in dormant tubers; for when the nitrogen of the nutrient solution was in the form of an ammonium salt no stimula-

<sup>6</sup> DAVIS, W. A., and DAISH, A. J. A STUDY OF THE METHODS OF ESTIMATION OF CARBOHYDRATES, ESPECIALLY IN PLANT-EXTRACTS. A NEW METHOD FOR THE ESTIMATION OF MALTOSE IN PRESENCE OF OTHER SUGARS. *Jour. Agr. Sci. [England]* 5: 437-468, illus. 1913.

<sup>7</sup> APPLEMAN, C. O. *Op. cit.*

tion occurred. Furthermore, no evidence was obtained to show that nitrate favored the accumulation of amino acid or amide nitrogen. On the contrary small concentrations of calcium nitrate strongly inhibited proteolytic enzyme activity and had a slight inhibitory effect upon the rate of the accumulation of amide nitrogen in expressed juice. Calcium nitrate was used because it proved to be less toxic than sodium or potassium nitrate when used as a single salt solution in cultural experiments. Although the specific effect of nitrates was not discovered by this study of the metabolism of the nitrogen compounds, evidence has been obtained that the cessation of the dormant period is dependent in part upon the activity of the proteolytic enzymes and the enzymes which convert amino into amide nitrogen.

Proteolytic enzyme activity may be measured by following in the juice the rate of accumulation of amino acid at low temperatures, provided the incubation period is short, but the results thus obtained are difficult to interpret because of the fact that the initial increase in amino-acid nitrogen is invariably followed by a decrease. The comparative proteolytic enzyme activity of samples of expressed potato juice may be followed with greater ease by adding casein to the juice. After the addition of casein the rate of the accumulation of amino-acid nitrogen was approximately a straight-line function of the time of incubation for periods as long as 96 hours at 37.5° C. The uniform rate of the accumulation of amino-acid nitrogen can not be explained by assuming that the original juice contained little protein, and hence that the addition of protein was required to demonstrate typical enzyme activity. On the contrary, large amounts of protein appeared to be present in the original juice, for upon removing the precipitate formed on heating the expressed juice, the total nitrogen was reduced to almost one-half.

Sure and Tottingham<sup>8</sup> have shown that amino acid may be converted into amide nitrogen. This phenomenon would serve to explain the decrease of amino acid which invariably followed the initial rise when expressed juice alone was incubated. The same phenomenon would also explain the apparent disappearance of amino acid during the first four weeks when tubers were stored at 5° C. Since the amide nitrogen content of the juice was invariably greater in nondormant than in dormant tubers there may be a possibility that amide nitrogen, possibly asparagine, is a factor upon which growth depends. The fact that the addition of asparagine to dormant tuber juice activates the carbohydrate hydrolytic enzymes but has no apparent influence upon nondormant tuber juice, tends to support this view.

#### SUMMARY

(1) The absorption of nitrates by potato tuber tissue abbreviated the dormant period, but under the same conditions ammonium salts did not affect it.

(2) There was a tendency for the amino and amide nitrogen to be greater in nondormant than in dormant tubers, but growth was not directly dependent upon the actual concentration of either of these compounds.

<sup>8</sup> SURE, B., and TOTTINGHAM, W. E. THE RELATION OF AMIDE NITROGEN TO THE NITROGEN METABOLISM OF THE PEA PLANT. *Jour. Biol. Chem.* 26: 535-548, illus. 1916.

(3) Proteolytic enzyme activity was more intense in the expressed juice of nondormant than in that of dormant tubers. When casein was added to the juice the rate of the accumulation of amino acid was a straight line function of the time of incubation.

(4) Evidence was obtained which indicated that when potato juice is incubated, amino acid nitrogen is converted into amide nitrogen.

(5) The addition of asparagine to dormant tuber juice activated the carbohydrate hydrolytic enzymes, but had no apparent influence upon nondormant tuber juice.

# THE DETERMINATION OF QUALITY IN SWEET CORN SEED BY MEANS OF THE OPTICAL MEASUREMENT OF LEACHED MATERIALS<sup>1</sup>

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## INTRODUCTION

In practice, injuries which impair the germination of seeds and the subsequent growth of the seedlings are ordinarily determined by means of actual yields from a field test, or, if this is not feasible, by the measurement of the heights, green weights, and dry weights of the seedlings as part of a greenhouse trial. The extent of such experiments and the time required for their completion imposes severe restrictions upon the number of samples which may be handled.

Several years ago, while working with sweet corn seed, the authors found it necessary to measure the injury caused by exposure to various temperatures. Limitations of time and space, as well as the large number of samples which required testing, made it imperative to devise a rapid and accurate method of determining viability and its relation to the subsequent vigor of growth in the seedling.

The senior author's observations made on seeds subjected to various temperatures and to chemicals in series of increasing concentration, showed that a marked correlation existed between the degree of injury to the seed and its susceptibility to fungous attack. The absence of fungous growth on the high quality control seeds and its progressive increase from the lower members in the series with only slight injury to the higher with increasing injury and final death, seemed to indicate that such growth is due to leachings from the seed. The measurement of such leached materials, inorganic and organic, crystalline and colloidal in nature, should serve as an index to the vigor of the protoplast and thus of the viability of the seeds. The effects of stimulation or injury on the permeability of the cell membranes have long been known. Osterhout (11)<sup>2</sup> and others in numerous experiments have recently measured the relation of conductivity and permeability to injury and death. They have found the electrical resistance of a plant to be an excellent indicator of what may be called its "normal condition of vitality." Any change in this condition is rapidly and positively shown by a change in electrical resistance. The method of Osterhout therefore received first attention. But before much progress had been made this was abandoned and others sought which would be more definitely applicable to the problem. It was essential that the methods used should measure not only the change in permeability of the membrane, but also the relative quantity of materials, crystalline organic and inorganic, and colloids, that are lost by diffusion through such changed membranes. In the conductivity method the permeability of the

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<sup>1</sup> Received for publication Apr. 25, 1927; issued August, 1927.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 166.

membrane is determined by the behavior of the electrolytes, whereas for this problem it seemed more appropriate to determine the permeability by measuring the inorganic and organic crystalloids and colloids. The former were measured by the use of the refractometer and the latter by the use of the nephelometer. After two years of investigation the refractive index and the colloidal index tests have proven very serviceable. These have their individual merits, but in practice supplement each other to a certain degree. The studies have been confined to sweet corn, but others working in the same laboratories have found the tests to be equally applicable to maize, garden peas, and radish.

#### LABORATORY METHODS

In the methods about to be described, the authors believe that both time and temperature of soaking the seed prior to the readings play an important part, but lack of space prevents giving this phase of the problem the attention it deserves. In experiments of this nature it is important to determine the point where pronounced differences in the colloidal index are obtained. After a number of trials 48 to 72 hours of soaking at 30° C. seemed to give the best results, and either one or the other of these periods was used in most of the subsequent readings. The 48-hour period at 30° appears to give the greatest differences in colloidal index when the germination of the corn has been in some way impaired. In such cases the leaching action is much more rapid than in normal corn and the best readings can be made within 48 hours. Sweet corn which is apparently normal in all respects leaches more slowly, and the longer period of soaking, 72 hours at 30°, gives the more reliable readings. In practice the length of the period can be determined with great ease by the appearance of the liquids after 24 hours leaching. If the colloidal solutions are quite opalescent the corn need not be soaked longer than 48 hours. On the other hand, clear or only slightly opalescent solutions indicate that 72 hours' soaking is desirable. Dispersions from badly injured seeds appear very turbid, and if many of them are in this condition the readings may be taken at 36 hours or sooner.

In order to obtain uniformity of results and freedom from the salts present in tap water, the seeds were soaked in distilled water. This brings up the question of the effect of distilled water on the protoplast and on leaching. André (1) observed that the seeds of beans and wheat soaked in distilled water for periods up to 281 days lost considerable amounts of nitrogen, phosphoric acid, and potash. True (13), True and Bartlett (14), Merrill (10), Hibbard (6), and others observe that distilled water is toxic to the roots of seedlings. Hibbard (6) states that where disease-free seed uniform in size, color, etc., is selected from the pure line offspring of one plant, there is as much variation in the behavior of the seedlings toward toxic substances in solution as in a mixed progeny from unknown sources. Hibbard states further that the harmful action of distilled water to seedlings is due not to a single predominant factor, but to a combination of many. These factors he conceives as forces bringing about a disturbance of the normal equilibrium of the various chemical and physical interactions within the organism and between it and its environment. Merrill (10) gives three reasons which may account

for the toxicity of distilled water to plants: (1) The lack of essential nutrients; (2) the presence of deleterious substances; and (3) the extraction of salts, or nutrient materials, from the organism immersed in distilled water. True (13) has made extensive observations concerning the relation between growth and the leaching of electrolytes from the roots of lupine seedlings grown in distilled water. He shows that with roots the rate of leaching is greatest at the end of 24 hours and progressively decreases with each 24-hour interval. The growth rate in this culture falls off in a manner suggesting a parallel with the decrease in resistance.

André (1), with seeds of wheat and garden beans, shows a leaching effect which is similar in some respects to the loss of electrolytes from the seedlings of True (13). It is the work of André and True which suggested a possible means of measuring the vigor of seeds by determining the soluble solids which leach from them in distilled water.

The proportions of corn and distilled water used in experiments of this character require careful consideration. In corn of high quality and consequently low permeability a ratio of 5 c. c. of distilled water per gram of corn is sufficient. In other cases 10 c. c. of distilled water per gram of corn is advisable. In all of their tests the authors used either 5 or 10 gm. of corn in 50 c. c. of distilled water. Due to the necessity of using whole grains it is virtually impossible to weigh out rapidly and accurately less than 5 gm. of corn.

In making the nephelometer test a proper standard of comparison is absolutely necessary. Kleinmann (7) has pointed out that the standard solution should be capable of remaining constant for several months. This is true of many inorganic sols, but care must be exercised with reference to color.

The colloids leached from the Country Gentleman and Narrow Grain Evergreen varieties of sweet corn are either clear or opalescent. Very rarely, and in the case of badly discolored and diseased specimens only, the liquid is somewhat yellowish. The best results were obtained, therefore, by the use of a slightly opalescent standard. After testing a considerable number of standards, including those used by Kober (8), Richards and Wells (12), and others, the authors found that chemically pure soluble starch fulfilled all requirements except one, namely, its keeping quality. This was overcome by making up a solution containing 0.5 per cent soluble starch and 0.5 per cent sodium toluene para sulphochloramid. The keeping qualities of this standard are excellent. Since the soluble starch obtained from the manufacturer varies in quality, care should be exercised to make the standard for a given series of experiments from the same lot.

In soaking samples of sweet corn the presence of bits of silks and chaff, pieces of pericarp and other detritus is practically unavoidable. Blowing and cleaning the samples on a screen previous to soaking removes only the coarser particles. Previous washing in distilled water also fails to eliminate them. The presence of these foreign, noncolloidal materials, together with the large particles in suspension due to coalescence, seriously affects the accuracy of the readings. Folin and Denis (5) have shown that the presence of large particles due to coalescence causes gross errors in the nephelometer readings. A series of tests by the authors shows that these errors could be greatly reduced by filtering the colloidal solutions through a porous filter paper.

The authors maintained a uniform procedure in preparing the samples. The ears in each case were shelled into a small box without either butting, tipping, or removing any of the diseased kernels. The kernels were well mixed, cleaned by screening and blowing, and then weighed out. Wide-mouthed bottles of 150 c. c. capacity were used for soaking the seed. The weighed samples were placed in these bottles, the distilled water added, and the bottles stoppered to prevent evaporation of the liquid. Each sample was then thoroughly shaken in order to remove air bubbles from around the kernels and likewise to immerse those which floated. The corn was leached in large, accurately controlled temperature cases. The bottles were removed at the end of the required period and the liquid of each decanted into a funnel lined with a porous filter paper. The filtrate was allowed to run into 100 c. c. test tubes and the readings taken as soon as sufficient liquid had filtered. In most instances duplicate samples were weighed from each ear and two readings taken from the respective filtrates.

The character of the experiments is such that both refractometer and nephelometer readings were taken for many of the samples. The technic described thus far proved applicable for both tests. Filtering the liquid is of course not necessary if only a refractometer reading is taken, as the index is affected only by the solutes.

All readings were checked by means of growth tests on greenhouse benches. A duplicate random sample of 20 kernels from each ear was planted in a single row  $\frac{1}{2}$  inch deep and 1 inch apart, the rows being 2 inches apart. Each fifth row was planted to a control consisting of a random sample showing 100 per cent rag-doll germination, and testing relatively free from rots and molds. All the ears in a given test were planted the same day. Measurements were taken in a single day at the age of about 18 days. The germination was computed to a percentage basis by assuming that if each of the 20 kernels produced a seedling, the germination was 100 per cent. The failure of one kernel to grow reduced this to 95 per cent, of two kernels, to 90 per cent, etc. Each seedling was evaluated 5 per cent. The height of each plant was the measured distance from the ground to the tip of the longest leaf. Averages were calculated by dividing the sum of the heights by 20. After they had been measured, the plants in each row were cut off at ground level and weighed. This total weight divided by 20 represented the average green weight. The average heights and green weights have been compared in each case with the respective average heights and green weights of the controls. The resulting increases or decreases were then compared with their respective instrument readings, and the correlation coefficients determined.

#### METHODS OF CALCULATION

The many thousands of observations necessitate presenting results in extremely condensed form. For this purpose much of the subsequent data have been calculated as correlation coefficients according to the formula of Pearson:

$$r_{xy} = \left[ \frac{(\sum d_x d_y)}{n} - w_x w_y \right] \left[ \frac{1}{\sigma_x \sigma_y} \right]$$

in which  $r_{xy}$  is the coefficient between the characters  $x$  and  $y$ ;  $d$ , the deviations;  $w$ , the correction factor;  $n$ , the number of observations;

and  $\sigma$ , the standard deviation. The probable errors of  $r_{xj}$  have been calculated from the formula

$$\text{P. E.}_r = \pm \frac{0.6745 (1 - r^2)}{\sqrt{n}}$$

In certain cases the standard deviations have been determined from the "short method" formula

$$\sigma = \sqrt{\frac{\sum (fD^2)}{n} - w^2}$$

in which  $f$  means the frequency,  $D$  means the deviation from the assumed mean, and  $w$  the correction factor.

The probable errors of the standards of deviation have been calculated from the formula:

$$E\sigma = \pm \frac{0.6745\sigma}{\sqrt{2n}}$$

#### THE REFRACTIVE INDEX TEST

The Abbé refractometer provides a rapid and highly accurate means of determining the specific gravity of a liquid and the percentage of total solids in solution. Bigelow and Fitzgerald (2), basing their investigations on Windisch's tables, have shown that the specific gravity and total solids in tomato pulp bear the same relation to each other as in beer and wine extracts. Since such proportions have been found to exist in these and in other instances, it may be assumed that the leachings from sweet corn kernels are proportional in the same manner. With this assumption a series of experiments was undertaken using a Bausch & Lomb Abbé refractometer.

Tables 1 and 2 give the results of preliminary tests on random samples of seed treated in different ways for the purpose of showing the reliability of the test. The sweet corn used in this test was a random sample of Country Gentleman showing good germination and almost complete freedom from rots or molds.

TABLE 1.—Comparison of the total solids in solution leached from sweet corn soaked in distilled water at various controlled temperatures for two different periods of time

Temperature at which corn was leached	Refractive index (25° C.)	
	After 24 hours	After 96 hours
° C.		
30	1.3345	1.3354
30	1.3345	1.3352
20	1.3342	1.3345
20	1.3342	1.3345
10	1.3341	1.3343
10	1.3340	1.3342
Distilled water.	1.3325	1.3325

From Table 1 it is evident that leaching is progressive both as to time and temperature. The experiment could not be continued beyond 96 hours, because of the fermentation occurring in the 30° C. sample.

In Table 2 a comparison is made between samples of killed and viable Narrow Grain Evergreen sweet corn taken from a single lot of seed. This corn tested 95 per cent germination and was free from disease. Twelve 25-gm. samples were weighed out and six of these subjected to a temperature of 100° C. for one hour in order to destroy life. The readings in Table 2 are the averages of eight replications. Each lot was leached in 50 c. c. of distilled water at the temperatures designated. The results show clearly that the rate of leaching is much more rapid in the killed seed and, likewise, that the rate of loss in both killed and viable samples increases progressively with the temperature.

TABLE 2.—*Comparison of the loss of soluble total solids from viable and killed seed of Narrow Grain Evergreen sweet corn after 48 hours leaching*

Condition of seed	Temperature at which seed was leached	Average refractive index (21.5° C.)
Killed.....	30	1.33600
Viable.....	30	1.33515
Killed.....	20	1.33545
Viable.....	20	1.33470
Killed.....	10	1.33460
Viable.....	10	1.33455
Distilled water.....		1.332845

### THE COLLOIDAL INDEX TEST

During the course of the earlier experiments it was noted that whereas in a few samples the liquid remained perfectly clear at the end of the soaking period, in the great majority it was more or less opalescent. Observations revealed that strong, vigorous growth was usually coincidental with a clear liquid, while the opposite was true of samples in which the liquid was opalescent. Test readings of such liquids were made with a Duboscq colorimeter converted into a nephelometer according to the method of Bloor (3). This instrument is capable of measuring with great precision the dispersity of a suspension such as is found when sweet corn kernels are treated with distilled water.

The corn was prepared as already described and the readings made with both tubes of the nephelometer nearly full and at exactly the same height, thus eliminating the necessity of volume corrections.

In order to determine whether colloids respond in a manner similar to the solids in solution, a number of experiments were undertaken, of which the results shown in Table 3 are representative. The samples for this test were prepared from the same lot and treated in the manner indicated in Table 1. At the close of the 66-hour period of soaking at 10°, 20°, and 30° C., respectively, the colloids increased

progressively with the temperature and had an extremely wide range. After 96 hours' soaking, however, the colloids as measured by the nephelometer had become nearly identical in quantity and the index in each case was very low. The respective suspensions gave visible evidence of the action of microorganisms.

It is quite likely that this is the principal reason for the lack of uniformity in some of the results given later. Subsequent work, not reported here, prevented such inconsistencies largely through the addition to the distilled water of a germicide that has no tendency to coalesce the colloids. In these investigations, however, the addition of a germicide has been avoided, as the primary object is to measure the normal leaching in sweet corn in no wise modified by the presence of a foreign substance, and its likely effect on changing the permeability of the membranes.

TABLE 3.—*Comparison of the loss of colloids from viable and killed seed of Country Gentleman sweet corn*

Temperature	Number of readings	Colloidal index (standard 30)	Time leached (hours)	Condition of seed
° C.				
30.....	4	12.40	66	Viable.
20.....	4	22.50	66	Do.
10.....	4	74.20	66	Do.
30.....	4	7.60	66	Killed.
20.....	4	13.00	66	Do.
10.....	4	43.20	66	Do.
30.....	4	2.32	96	Viable.
20.....	4	3.90	96	Do.
10.....	4	16.50	96	Do.
30.....	4	2.25	96	Killed.
20.....	4	2.40	96	Do.
10.....	4	5.85	96	Do.

Other methods of controlling the growth of microorganisms have suggested themselves, the most promising being the leaching for longer periods at temperatures which inhibit or prevent their growth, and yet are not injurious to the seeds. (Compare Tables 1, 2, and 3.)

Under conditions favorable to the rapid development of organisms, it may be surmised that the colloidal index becomes largely a measure of the dispersity of the microorganisms. That this is not the case is indicated by the samples in Table 3 which were leached at 10° C., a temperature not favorable for the rapid growth of bacteria and fungi. The relatively low colloidal indices of the sweet corn soaked at 10° for 96 hours can be interpreted as due solely to the presence in such samples of membranes impaired by disease or otherwise and the consequent free diffusion of cell colloids through them. This view is further supported by comparisons of the colloidal indices of killed and viable seeds in Table 3. Here the corn subjected to 100° for one hour was killed and a change occurred in the protoplast, including the organic membranes. At the same time, at least partial sterilization is indicated. Nevertheless, the colloidal indices of the viable, unsterilized samples are consistently higher. Table 3 represents only one of many similar tests, all of which have the same

trend. However, there can be no doubt that microorganisms do affect the suspensions. Whether the cause is physical (such as heat), chemical, or pathological, the weakened membrane permits the colloids and soluble solids to diffuse through it with greater freedom. Concomitantly, the growth of microorganisms, because of the greater abundance of food, has an upward tendency. This increased growth of microorganisms may actually raise the colloidal index by decreasing the colloids in suspension. This explains some of the discrepancies which will be noted later.

It is quite probable that improved technic and the use of more sensitive instruments such as the photometer, instead of the nephelometer, will further increase the reliability of this test. The authors have realized from the start that the use of filter paper in preparing the samples for nephelometer readings is open to criticism on the score that some of the suspension particles would be retained. The question is, as mentioned previously, whether filter paper is the source of greater errors for such uses as here outlined than those which are known to result from coalesced colloidal particles and the unavoidable presence of dust and refuse from the samples. In certain instances which, however, have not been observed more than a half-dozen times in thousands of readings made, the leachings are viscous and approach the nature of a gel. Such liquids are virtually unfilterable, and in cases where the index of the filtrate alone is taken it is invariably too high. Ears Nos. 51 and 55 in Table 10 are typical of this condition.

In this connection an error due to the instrument itself may be mentioned. Kober (8) and Kober and Egerer (9) show that the amounts of precipitate in solution are not exactly in inverse proportion to the calibration on the scale. In cases where the density of the precipitate in the standard solution and the unknown are within 10 per cent of each other (about 20 per cent when the depths of the liquids are 60 mm.) accurate readings can be taken directly from the scale. If greater differences occur, corrections are necessary. For this purpose Kober (8) proposes a formula which has been used successfully by other workers. Since the variations in the case of sweet corn samples are much wider than those encountered in the usual laboratory analytical work, it is obvious that very wide differences occur between the standard and unknown. In a relatively dense hydrosol, such as the standard used by the authors, the readings for the unknown solutions which contain a smaller percentage of colloids are subject to a greater error than in cases where the standard and unknown are within 10 to 20 per cent of each other. Kober (8) shows that when the ratios between standard and unknown are within 10 per cent of unity, the edestin curve and the hypothetical curve (which is inversely proportional to the concentration of the solutions) are within 1.5 per cent of each other. It is evident that if very accurate readings are required it is necessary either to adjust the volumes of the solutions as indicated by Richards and Wells (12), to use the formula of Kober (8) and Kober and Egerer (9), or to vary the concentration of the standard.

The application of Kober's formula in this instance is open to question because of the fact that the composition of the unknown consists of a mixture of colloids while the standard is a starch solution. The adjustment of the volumes, or perhaps better still, the use of the

standard adjusted to varying heights, is the safer course. In the present paper the authors have avoided corrections because it is very probable that the same purpose can be accomplished by the use of a single standard set at a given point (30 mm.). Of course, there is a sacrifice to accuracy, but it is doubtful whether absolute accuracy is essential in experiments such as this, where the range is determined only by the limits of the nephelometer scale. It is well to remember that when hundreds of samples in a series are ready to read, the work must be completed within a few hours, otherwise serious errors will occur on account of the differences in time for diffusion between the first and the last members of each series. Where the filtered samples are held there is danger of coalescence.

#### THE EFFECT OF PHYSICAL INJURIES ON THE PROTOPLAST INDUCED BY ARTIFICIAL CURING, AS MEASURED BY THE QUANTITY OF LEACHED MATERIAL

Although some data have been presented which indicate that both the refractometer and nephelometer tests are delicate in the measurement of the quantity of material leached from seeds differently treated, nevertheless the authors have deemed this phase of the investigation sufficiently important to add a series of tests on viable, yet injured seeds. As previously stated, this study is the offshoot of an experiment to determine the heat resistance of immature sweet corn.

Country Gentleman sweet corn was picked at three different stages of maturity and dried in a specially constructed seed-corn drier provided with wire shelves at different heights so that three temperatures, 47°, 43°, and 37° C., called high, medium, and low, respectively, in Table 4, could be maintained. Circulation was provided by means of a forge blower, while the heating arrangements consisted of large electrical heating units located at the base of the drier.

TABLE 4.—*Effect of physical injuries caused by artificial curing on the quantity of material leached*

Temperature in drier	Time in drier (hours)	Initial moisture (per cent)	Greenhouse test			Refractive index	Colloidal index
			Mean germination (per cent)	Mean increases over controls			
				Green weight (gm.)	Height (mm.)		
High.....	48	69	3.8	-0.16	-63.8	1.33505	2.18
Medium.....	48	69	17.5	-0.19	-66.2	1.33514	4.09
Low.....	48	69	41.2	-0.19	-35.3	1.33476	6.60
High.....	96	69	0	-0.26	-112.2	1.33560	1.98
Medium.....	96	69	12.5	-0.25	-85.2	1.33491	3.09
Low.....	96	69	33.8	-0.22	-48.4	1.33482	5.56
Room.....		69	70.0	-0.02	11.1	1.33443	12.30
High.....	48	44.2	98.8	0.30	61.8	1.33410	27.72
Medium.....	48	44.2	100.0	0.18	53.6	1.33408	30.65
Low.....	48	44.2	93.8	0.28	52.0	1.33414	21.72
High.....	96	44.2	71.2	-0.01	-11.8	1.33442	9.72
Medium.....	96	44.2	98.8	0.26	66.0	1.33422	18.05
Low.....	96	44.2	83.8	0.21	45.7	1.33424	20.82
Room.....		44.2	97.5	0.66	92.7	1.33400	51.30

The initial moisture contents of the two pickings reported in Table 4 were 69 per cent and 44.2 per cent, respectively, computed on the dry basis. Duplicate readings were taken of duplicate samples from each ear. Thus the figures in Table 4 are the averages of eight readings. The samples were soaked at 30° C. for 48 hours, the proportions being 5 gm. of sweet corn in 50 c. c. of distilled water. The nephelometer standard was set at 30 mm. and the refractive indices were read at 21°.

In Table 4 the corn with 69 per cent initial moisture shows clearly that the quantity of leached material increases in relation to the degree of injury to the protoplast as affected by temperature and by the period of exposure. The injury done by such treatments is clearly manifested by the germination and growth of the seedlings and the refractive and colloidal indices. The latter, it will be noted, are in close agreement with the former. In cases where the initial percentage of moisture is 44.2 the agreement is not so close, but this is exactly what is to be expected in a case where the external factors have little or no injurious effect. The data in Table 4 show how remarkably sensitive the protoplast really is and that even slight changes in external conditions are sufficient to modify the permeability and the subsequent growth.

The growth records for the entire series have been correlated with the respective refractive and colloidal indices obtained from identical samples just previous to planting, and are given in Table 5. The frequency distribution in Table 6, which also gives the methods of calculation in detail, shows the close agreement between permeability and subsequent growth.

TABLE 5.—Correlations between seedling characters and instrument readings for 116 ears of Country Gentleman sweet corn cured by artificial heat

Seedling characters	Refractive index (21° C.)	Colloidal index (Standard 30 mm.)
Per cent germination.....	$r_{xy} = 0.592 \pm 0.041$	$r_{xy} = 0.634 \pm 0.037$
Difference in height as compared with control.....	$r_{xy} = .360 \pm .055$	$r_{xy} = .680 \pm .034$
Difference in green weight as compared with control.....	$r_{xy} = .343 \pm .055$	$r_{xy} = .693 \pm .033$

TABLE 6.—*Correlation between the colloidal index and the difference in green weight of seedlings from sweet corn cured by artificial heat*

Green weights (gm )																										
Colloidal indices	Less than control										More than control										fy	dy	Σdx	Σdx dy	fdy	fd'y
	0	60	0.50	0.40	0.30	0.20	0.10	0	0.10	0.20	0.30	0.40	0.50	0.60	0.70											
	.51	.41	.31	.21	.11	.01	.09	.19	.29	.39	.49	.59	.69	.79												
1-3.....	2		3	15		7	3									30	0	94								
4-6.....	1	2	5	9		3	5	1	1							27	1	89	89	27	27					
7-9.....	1			2		6	4		1	4	1		1			20	2	109	218	40	80					
10-12.....						1			2	1	2	3				9	3	74	222	27	81					
13-15.....							1	2	1	1		1		1		7	4	54	216	28	112					
16-18.....								2	2							4	5	26	130	20	100					
19-21.....							1	2					2			5	6	39	234	30	180					
22-24.....										1						1	7	8	56	7	49					
25-27.....										1	1					2	8	17	136	16	128					
28-30.....										2						2	9	16	144	18	162					
31-33.....										1	1	1				3	10	27	270	30	300					
34-36.....															1	1	11	13	143	11	121					
37-39.....												1		1		2	12	22	264	24	288					
40-42.....											1					1	13	9	117	13	169					
43-45.....																0	14									
46-48.....							1									1	15	6	90	15	225					
49-51.....																1	1	16	13	208	16	256				
x.....	4	2	8	26	17	14	8	7	11	6	6	3	2	2	2	116		616	2537	322	2278					
dx.....	0	1	2	3	4	5	6	7	8	9	10	11	12	13	Totals.											
fdx.....		2	16	78	68	70	48	49	88	54	60	33	24	26		616										
fd'x.....		2	32	234	272	350	288	343	704	486	600	363	288	338		4300										

$$wx = \frac{616}{116} = 5.310 \quad w^2y = 7.706 \quad \frac{fd^2y}{n} - w^2y = 11.932 \quad r_{xy} = \frac{7.131}{\sigma_x \sigma_y} = 0.69r$$

$$w^2x = 28.196 \quad \frac{fd^2x}{n} - w^2x = 8.873 \quad \sigma_y = \sqrt{11.932} = 3.454 \quad \text{P. E.}_r = \pm 0.033$$

$$wy = \frac{322}{116} = 2.776 \quad \sigma_x = \sqrt{8.873} = 2.979 \quad \frac{\Sigma dx dy}{n} - wxwy = 7.131$$

In Table 5 the values of  $r$  indicate that there is a somewhat close degree of association between the colloidal index and the seedling behavior than in the case of the refractive index. This is to be expected for two reasons: (1) The content of soluble solids in seeds is relatively low and the Abbé instrument is not entirely reliable for readings in the fourth decimal place; and (2) since the colloids play by far the more important physiological rôle, the quantitative loss as measured by the nephelometer is a closer index of injury to the protoplast. This lack of entire agreement between the refractive and colloidal indices is brought out in Table 7, which has been calculated from a series of 500 ears not injured by heat. The correlation coefficient for this distribution is  $0.713 \pm 0.015$ , showing that there is a very significant tendency for the colloids to vary in relation to the total solids, but on account of the limitations of the Abbé instrument and the small percentages of soluble solids, the agreement between them is by no means perfect.

TABLE 7.—Correlation between refractive and colloidal indices in a series of 500 ears of Country Gentleman sweet corn selected from the field

Refractive indices	Colloidal indices																				f <sub>0</sub>
	1-4	5-8	9-12	13-16	17-20	21-24	25-28	29-32	33-36	37-40	41-44	45-48	49-52	53-56	57-60	61-64	65-68	69-72	73-76	77-80	
1.3358																					1
1.3357		1																			0
1.3356																					0
1.3355																					0
1.3354		1																			2
1.3353		1																			2
1.3352	1																				1
1.3351	1	1																			3
1.3350	1	3	2	3	1		1														11
1.3349	3	6	5	3	4	2	1	2	1			2								1	30
1.3348		2	18	9	6	6	2	2	1	3											48
1.3347			2	4	14	9	2	3			1										35
1.3346					11	12	7	6	3	4	1	2		1				1			45
1.3345			2	2	10	7	14	12	13	5	5	4		1	1						75
1.3344			1	1	7	5	5	11	7	7	6	3	6	1	1		1		1	2	65
1.3343					2	6	4	8	17	15	3	5	9	1	5	1	1	1		1	79
1.3342						1	2	4	3	10	9	11	11	5	7	10	3		3	5	84
1.3341											3	1		1	2		2	2	1	5	17
1.3340																1				1	2
x.....	6	15	32	27	55	48	37	44	48	39	29	26	27	9	15	12	8	3	5	15	500

$$r_{xy} = 0.713 \pm 0.015.$$

#### INFLUENCE OF THE STRUCTURE OF THE PERICARP ON PERMEABILITY

The question of the influence of the pericarp on the rate of diffusion of colloids naturally presented itself. Koritz<sup>3</sup> has measured the resistance of the pericarp of sweet corn to puncture. He found that there is no uniformity in resistance to puncture either among kernels on a single ear or between different ears in the same open-pollinated strain of sweet corn. This is to be expected on account of the hybrid nature of sweet corn. His investigations, however, lead to the conclusion that quite the opposite is true in  $F_3$  self-pollinated, apparently homozygous lines. Here the resistance to puncture was uniform, both as to individual kernels on the ear and between different ears in the same strain. As Koritz and the authors collaborated by using the same samples in this series and made their respective observations at the same time, it is possible to compare the two sets of data.

The coefficient  $r_{xy} = 0.104 \pm 0.061$  is obtained when the penetration values of the 98 ears mentioned in Table 10 are correlated with the corresponding colloidal indices. This coefficient is less than twice the probable error and its significance is very slight. It was deemed advisable to confirm these observations with readings from pure line strains, especially in view of Hibbard's (6) observations that there is as much variation in behavior toward distilled water within pure lines as in a mixed progeny.

A further comparison was made between 68 ears selected from  $F_3$  pure lines of Narrow Grain Evergreen sweet corn and 78 ears from  $F_3$  pure lines of Country Gentleman sweet corn. Owing to the scarcity of available material it was impossible to secure sufficient ears from a

<sup>3</sup> KORITZ, L. A. A STUDY OF THE PHYSICAL PROPERTIES OF THE SWEET CORN PERICARP. [Unpublished thesis. Copy on file, Univ. Ill. Libr.]

single strain. Several strains were utilized and the penetration values with the respective colloidal indices of a few are given in Table 8. It will be noted that the penetration values are remarkably uniform, while the colloidal indices vary widely. These data indicate that the structure of the pericarp, as measured by a puncturing device, is not correlated with the rate of diffusion which varies independently. This is shown further by the coefficients of correlation between the colloidal indices and the respective penetration values. For the  $F_3$  Narrow Grain Evergreen strains  $r_{xy} = 0.177 \pm 0.056$  and for the  $F_3$  Country Gentleman strains  $r_{xy} = 0.022 \pm 0.039$ . Neither of these coefficients has much significance.

TABLE 8.—*The relation between penetration values and colloidal indices in  $F_3$  self-pollinated strains of sweet corn*

Strain number	Variety	Penetration value	Colloidal index	Strain number	Variety	Penetration value	Colloidal index
306-19	Country Gentleman	78.2	17.2	207-446	Narrow Grain Evergreen	43.9	37.5
		76.0	18.4			43.9	24.6
		76.0	12.4			47.7	30.0
		76.6	16.6			38.1	47.5
		76.0	40.2			47.7	21.2
		76.0	52.1			43.9	20.2
		77.7	19.4			47.7	38.6
334-82	Country Gentleman	46.4	52.2	248-493	Narrow Grain Evergreen	44.1	19.8
		45.3	37.0			41.2	32.0
		45.9	39.9			41.1	17.9
		47.5	28.3			41.2	37.2
		47.5	33.2			41.2	36.5
		47.9	51.2			46.0	6.8
		47.5	58.6			50.4	22.9
		47.7	27.1			41.1	32.8
		47.5	40.2			41.2	69.2
		47.5	15.2			43.0	26.6

The coefficients of correlation for the entire  $F_3$  population have been summarized in Table 9. These coefficients are just as significant as in the case of the open-pollinated strains, indicating that permeability is just as variable. It is interesting to note that, as shown in Table 8, there is a tendency for certain strains to have a higher range of colloidal indices than others. This brings up the question of the relation of colloidal indices to disease susceptibility found in homozygous strains.

#### THE VALUE OF THE NEPHELOMETER TEST UNDER PRACTICAL CONDITIONS

For the purpose of further determining the reliability of the nephelometer test, a series of 98 ears was selected from a single open-pollinated strain of Country Gentleman sweet corn. These ears were classified according to the rag-doll test, but only two of the largest classes are given in Table 10. When the colloidal indices are compared with the respective rag-doll and greenhouse percentages of germination, it will be found that there is very close agreement. Occasionally an ear found good by the rag-doll test is pronounced poor by its low colloidal index. Greenhouse and field tests almost invariably support the quality as indicated by the nephelometer test of such an ear.

TABLE 9.—Correlations between seedling behavior and colloidal indices in a population of *F<sub>2</sub>* inbred strains of two varieties of sweet corn

Seedling character	Colloidal index of—	
	Narrow Grain Evergreen	Country Gentleman
Germination (greenhouse).....	0.554±0.057	0.504±0.057
Difference in height as compared with control.....	.414±.067	.410±.064
Difference in green weight as compared with control.....	.408±.068	.444±.061

TABLE 10.—Relation between seedling behavior and colloidal index in part of a 98-ear series of Country Gentleman sweet corn when rag-doll readings are constant (100 per cent)

Ear No.	Colloidal index (standard 30 mm.)	Rag-doll test—seedlings diseased (per cent)	Greenhouse test			Ear No.	Colloidal index (standard 30 mm.)	Rag-doll test—seedlings diseased (per cent)	Greenhouse test		
			Germination (per cent)	Average increases over control					Germination (per cent)	Average increases over control	
				Height (mm.)	Green weight (gm.)					Height (mm.)	Green weight (gm.)
2.....	27.3	None	95	-19.0	-0.37	11.....	51.0	None.	100	-42.1	-0.50
13.....	38.4	None.	100	-18.8	-0.47	12.....	26.1	None	100	-22.4	-0.24
3.....	37.2	None.	100	9.0	0.03	4.....	30.6	None	95	17.5	0.15
6.....	25.8	None.	100	32.5	0.17	15.....	32.4	None.	100	-23.0	-0.20
28.....	32.7	None.	100	-3.2	-0.16	8.....	26.1	None	100	45.8	0.93
19.....	26.7	None.	90	-4.6	0.10	17.....	24.6	None	95	35.0	0.67
21.....	9.3	None	95	14.0	0.15	20.....	38.4	None.	95	9.8	0.36
1.....	30.3	None	90	-57.2	-0.80	59.....	9.6	40	50	-167.1	-1.54
7.....	24.3	None.	100	29.7	0.56	60.....	6.0	40	70	-113.9	-1.10
24.....	42.0	None.	100	33.7	0.08	53.....	11.7	40	90	-32.3	-0.53
5.....	45.0	None.	100	34.7	0.47	57.....	3.3	40	60	-77.2	-0.62
14.....	40.5	None.	100	10.8	0.28	47.....	7.5	40	50	-118.3	-1.06
26.....	28.8	None.	100	27.2	0.14	56.....	6.3	40	70	-53.6	-0.35
27.....	75.6	None.	85	-35.4	-0.29	49.....	12.6	40	70	-75.1	-0.78
18.....	49.2	None.	95	2.6	0.12	54.....	1.5	40	35	-136.8	-1.00
22.....	36.1	None	100	2.8	0.08	50.....	6.0	40	45	-121.4	-0.96
25.....	34.2	None.	100	31.8	0.07	55 <sup>a</sup> .....	18.0	40	70	-41.6	-0.25
9.....	36.6	None	90	39.1	1.75	52.....	50.4	40	85	11.8	0.25
16.....	39.0	None.	100	18.3	0.50	58.....	4.2	40	65	-44.7	-0.18
23.....	38.4	None.	85	4.5	0.28	51 <sup>a</sup> .....	51.6	40	85	-4.2	-0.03
10.....	65.7	None.	100	52.4	1.85	48.....	15.6	40	60	-65.3	-0.33

<sup>a</sup> Filter very slowly.

NOTE.—Colloidal index readings from 10 gm. corn in 50 c. c. distilled water soaked at 30° C. for 72 hours.

It is of interest to note that samples taken from the 28 ears which show 100 per cent germination and are free from disease, as shown by rag-doll germination, are not in all cases in agreement with the nephelometer test. The colloidal index, 9.3, for ear No. 21, for example, indicates a poor quality ear; but by rag-doll and greenhouse tests it is of good quality. The opposite is true of ear No. 27. On the other hand, in the samples from ears which show 100 per cent germination and are 60 per cent disease-free, the agreement with the nephelometer test is much closer. The tendency for the green weights and heights of seedlings to agree more closely with the colloidal indices when the permeability of the protoplast has been increased through injury or disease, is one which the authors have frequently observed. The cause of this tendency is probably the earlier inhibition of measurable growth in cases where the injuries

due to disease are severe. If the weakness is slight, as in the case of the 28 ears which showed 100 per cent rag-doll germination and were free from disease, early seedling growth gives no visible evidence and there results an apparent disagreement with the colloidal indices. It is better to consider ears showing this discrepancy of questionable quality and to eliminate them. Further tests in the field will almost invariably show that such ears are susceptible to later injuries and disease. In some very favorable seasons they will do well, but under adverse conditions will rapidly deteriorate. Similar results were obtained by Burkholder (4) with beans. The same tendency has been observed on a much larger scale in connection with the tests covered by Table 16, which will be referred to in their place.

The data for the entire series of 98 ears are given as frequency distributions in Tables 11, 12, and 13. The respective coefficients of correlation have been calculated. In Table 11 the colloidal indices and percentages of greenhouse germination are in fairly close agreement, which confirms the observations made from Table 10. The coefficient of  $0.498 \pm 0.051$  from Table 11 shows that a close degree of association exists.

In Table 12 the seedling heights and the colloidal indices are similarly compared, and from the coefficient of  $0.534 \pm 0.049$  it is evident that the association is even closer, due to the fact that most of the seedlings are smaller than the controls. This shows further that in cases where the protoplasts are severely injured seedling growth will be in closer accordance with the colloidal index than it will in cases where the protoplasts are slightly injured.

Table 13 contains a comparison between the colloidal indices and the green weights of the seedlings from which a coefficient of  $0.547 \pm 0.048$  is obtained. Here again there exists the same relationship as shown in Table 12, which further supports the conclusions already drawn.

TABLE 11.—*Correlation between the greenhouse germinations and colloidal indices in a single strain of Country Gentleman sweet corn*

Colloidal indices	Per cent germination																			fy
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	
1-4							2	1	1	1		1	1	1				1		9
5-8		2					1	1	1	1	3		3		2	1		1	2	18
9-12		1						1	1		1			1	2	2			2	15
13-16								1					1	1				1	2	13
17-20								1							4				1	9
21-24																			1	4
25-28																			1	7
29-32																			1	4
33-36																			1	2
37-40																			1	7
41-44																			1	1
45-48																			1	1
49-52																		2	1	4
53-56																			1	1
57-60															1					1
61-64																				
65-68																				1
69-72																				
73-76																		1		1
77-80																				
$\Sigma$	0	3	0	0	0	0	3	4	2	2	4	1	5	3	10	3	0	7	8	33
$\Sigma x$																				98

$$r_{xy} = 0.498 \pm 0.051$$

TABLE 12.—Correlation between heights of seedlings and colloidal indices in a single strain of Country Gentleman sweet corn

Colloidal indices	Heights of seedlings (mm.)													Σy
	Less than control										More than control			
	250-226	225-201	200-176	175-151	150-126	125-101	100-76	75-51	50-26	25-1	0-24	25-49	50-74	
1-4		1	1	2	2		1	1	1					9
5-8	1	1	1	4		5		1	3	2				18
9-12		1		2			5	1	3		1	2		15
13-16		1						5	1	5		1	1	13
17-20							1	1	3	1	2	1		9
21-24										1	1	2		4
25-28										4	1	3		7
29-32								1		1	1	1		4
33-36										1	1	1		2
37-40										1	5	1		7
41-44										1				1
45-48												1		1
49-52									1	1	2			4
53-56											1			1
57-60						1								1
61-64														
65-68													1	1
69-72														
73-76									1					1
77-80									1					1
Σx	1	4	2	8	2	6	7	10	13	18	13	13	1	98

$r_{xy} = 0.534 \pm 0.049$

$$r_{xy} = 0.534 \pm 0.049$$

TABLE 13.—Correlation between green weights of seedlings and colloidal indices in a single strain of Country Gentleman sweet corn

Colloidal indices	Green weights of seedlings (gm.)																			fx	
	Less than controls										More than controls										
	2.0-1.9	1.8-1.7	1.6-1.5	1.4-1.3	1.2-1.1	1.0-0.9	0.80-0.70	0.60-0.50	0.40-0.30	0.20-0.10	0.0-0.10	0.20-0.30	0.40-0.50	0.60-0.70	0.80-0.90	1.00-1.10	1.20-1.30	1.40-1.50	1.60-1.70		1.80-1.90
1-4	1	1		2	1	1	1	1	1					1							9
5-8		1	4	2	2	2			2	1		1									18
9-12		1	1		1	1	5	3						1							15
13-16	1				1	1	2	1	1	4	2			1							13
17-20								2		3											9
21-24												2		1							4
25-28										1	1	1		1							7
29-32							1			1	1	1		1	1						4
33-36										1	1										2
37-40								1			1								1		7
41-44											1										1
45-48													2	2							1
49-52								1													4
53-56												1	1								1
57-60							1					2	1								1
61-64																					
65-68																			1		1
69-72																					
73-76									1												1
77-80																					
fx	2	3	5	4	4	5	12	9	7	13	12	11	3	5	1	0	0	0	1	1	98

$$r_{xy} = 0.547 \pm 0.048$$

## THE RELATIVE VALUE OF THE OPTICAL TESTS IN RELATION TO GERMINATION AND SEEDLING VIGOR

It will be noted that the correlation coefficients exhibit a considerable degree of variation. The significance of such variation may be explained, first, on the basis that the number of seeds used in making both growth and instrument readings is relatively small; second, on the basis that in the better seeds the dispersity of the colloids is extremely high and, consequently, for reasons which have already been discussed, the reliability of the readings on the nephelometer scale rapidly decreases. This lack of reliability where the dispersity is high in no wise affects the usefulness of this test, since before this condition is reached the seeds are of a superior class.

Further evidence in support of the nephelometer test is given in the frequency distribution as shown in Table 6. The variability of the colloidal indices increases in relation to the increases in green weight, and this is further strikingly supported by the behavior of the untreated series of ears in Table 13. Comparisons of the colloidal indices with germination as shown in Table 11, and with the heights of seedlings, as shown in Table 12, still further support the nephelometer test.

In order to determine precisely the extent of the variability in both the nephelometer and refractometer tests, the readings from two large series have been classified and the standard deviations ( $\sigma$ ) calculated and presented in Tables 14 and 15. The values of  $\sigma$  for series 2, shown in Table 14, are given for both the rag-doll and greenhouse germinations. In this case the percentages represent readings from the same ears. No rag-doll records are given for series 1. It was impossible for the authors to secure enough ears in each 10 per cent class as here noted to give a reliable standard deviation. In cases where the number was not sufficient, the classes were omitted. A sufficient number of classes are represented, however, to show the trend distinctly.

The deviations for the colloidal indices in series 1 when arranged according to the percentages of greenhouse germination increase gradually up to 90 per cent. Beyond this point the deviation more than triples, which again shows that superior seed has a low colloidal leaching. This is not entirely supported by the distributions as shown in Tables 12 and 13. These tables show that a number of seeds with low colloidal indices gave high growth tests in the greenhouse. This apparent discrepancy between low colloidal index and high seedling growth vigor in the greenhouse, when followed in the field, vastly increases the claim of reliability of the nephelometer test. Field tests will show that seeds apparently vigorous in the germinator and in the greenhouse will fail to maintain this standard in the field. This is especially true when conditions are unfavorable.

In series 2 the trend is very similar, but more gradual. The rag-doll readings in series 2 show that the deviations in colloidal index become large while the germination is still extremely low, but this would be expected from the known unreliability of the rag-doll test.

TABLE 14.—*The standard deviations of the optical readings in relation to germination*

Germination (per cent)	Colloidal index of—		Refractive index of—	
	Series 1	Series 2	Series 1	Series 2
Greenhouse:				
0-10.....	1.614±0.140		0.0006540±0.0000600	
10-20.....	1.896±.404		.0003162±.0000533	
20-30.....	1.416±.390			0.0001939±0.0000414
30-40.....	2.448±.389		.0001662±.0000264	.0001707±.0000271
40-50.....				.0001920±.0000276
50-60.....				.0001925±.0000211
60-70.....		11.412±1.217	.0000980±.0000209	.0002450±.0000261
70-80.....	2.616±.394	12.240±.973	.0001662±.0000250	.0001986±.0000158
80-90.....	3.444±.411	14.880±.703	.0001732±.0000206	.0002532±.0000120
90-100.....	12.528±1.073	17.760±.502	.0002456±.0000210	.0002149±.0000607
Normal rag doll:				
0-10.....				
10-20.....				
20-30.....				.0002724±.0000392
30-40.....		6.880±.989		.0003645±.0000524
40-50.....		18.620±2.374		.0002771±.0000353
50-60.....		16.772±1.885		.0002728±.0000307
60-70.....		20.952±1.766		.0002663±.0000224
70-80.....		13.996±.926		.0002161±.0000143
80-90.....		16.496±.775		.0002315±.0000109
90-100.....		17.224±.530		.0002256±.0000609

TABLE 15.—*The standard deviations of the optical readings in relation to green weights and heights of seedlings*

	Colloidal index of Series 1	Refractive index of Series 1
Green weight (in grams) as compared with controls:		
—0.60 to —0.41.....	2.064±0.402	
—0.40 to —0.21.....	1.818±.149	0.0007142±0.0000584
—0.20 to —0.01.....	3.987±.342	.0003958±.0000339
0 to 0.19.....	5.424±.480	.0002248±.0000277
0.20 to 0.39.....	10.731±1.241	.0001846±.0000214
0.40 to 0.59.....	9.810±1.560	.0001572±.0000250
Height (in millimeters) as compared with controls:		
—140 to —151.....	1.479±.204	.0004230±.0000582
—100 to —61.....	1.926±.192	.0007734±.0000769
—60 to —21.....	2.682±.294	.0003864±.0000423
—20 to 19.....	10.419±1.205	.0002916±.0000337
20 to 59.....	7.185±.831	.0001912±.0000221
60 to 99.....	9.300±1.230	.0001442±.0000191

In Table 15 the standard deviations of the colloidal indices are arranged with reference to the green weights and seedling heights. In both cases deviations become larger as the vigor of the protoplast increases. There is some tendency for the deviations to decrease where growth is exceptionally vigorous, but this could not be confirmed on a larger scale because of the extreme scarcity of such ears.

When the coefficients of correlation for three different series are arranged in an ascending order with respect to their mean percentages of germination (Table 16), it will be found that the correlation coefficients for the colloidal indices decrease. This is due simply to the greater variability which is always found where the protoplasts are only slightly injured and emphasizes the limitations of seedling tests, whether rag-doll or greenhouse. Table 16 also confirms in a very decided way the data in Tables 14 and 15.

The corresponding deviations for the refractive index readings in series 1 and 2 are also compared in Tables 14 and 15. In series 1

there is some tendency for the deviations to decrease in relation to the increased vigor of the seedlings, but this is not sufficiently definite to be taken into consideration. In series 2, Table 14, the deviations vary but not in a definite direction. The authors believe that the variation in size of the standard deviation with respect to the refractive indices is largely due to experimental error. It is known that the soluble solids consist mainly of sugars and a small proportion of electrolytes. The total percentage is small and the range of refractive indices is, therefore, somewhat constricted, 1.3340 to 1.3360, with the larger part of the readings falling between 1.3343 and 1.3346. When this is coupled with the fact that the fourth decimal place is merely an approximation in the Abbé refractometer, it is evident that the standard deviations are largely a matter of chance.

This absence of greater variability at one end of the scale than at the other is a valuable characteristic, furnishing a check on the colloidal indices. The association between the two tests is close, as indicated by the high coefficient obtained from Table 7.

TABLE 16.—*The relation between the mean percentage of greenhouse germination and the nephelometer test*

Source	Greenhouse tests			
	Correlation coefficients			Mean germination (per cent)
	Germination (per cent)	Height	Green weight	
Table 5.....	0.634±0.037	0.680±0.034	0.693±0.033	55.8
Tables 11, 12, 13.....	.498±.051	.534±.049	.547±.048	76.5
500 ears, not previously given.....	.374±.026	.341±.027	.313±.027	86.5

## SUMMARY

The permeability of the protoplast to colloids may be measured by their dispersity in distilled water leachings.

The healthy and vigorous protoplast allows only small quantities of colloidal materials to leach through its membranes.

Injury or disease may result in physical and chemical changes within the protoplast and in a weakening of the semipermeable properties of its membranes.

The vigor and subsequent growth of the seedling, aside from its genetic constitution, is determined by the state of the protoplast.

The total soluble solids in distilled water leachings as determined by the refractive index are likewise a measure of permeability. Because of the small percentage of total solids present and the limitations of the instrument this test is not quite as responsive as is the colloidal index.

The total leachings from the protoplast are in no wise related to the resistance of the pericarp to puncture.

Neither the rag-doll nor growing tests, whether conducted in the greenhouse or in the field, measure a weakened condition of the protoplast as conclusively as the optical tests.

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# FACTORS INFLUENCING THE LOSS OF IODINE FROM IODIZED SALT<sup>1</sup>

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## INTRODUCTION

The prevalence of goiter in certain sections of the United States has resulted in the general use of iodized salt for both man and animals. The State of Montana lies in one of these so-called "goiterous" regions, and in order to prevent goiter and its associated conditions of weakness in farm and ranch animals, the veterinary department has recommended the use of stock salt containing 1 ounce of potassium iodide to 100 pounds of the salt.<sup>3</sup> This salt is frequently prepared in large quantities and stored in convenient places on the range. After it has been in storage for several months or longer, stockmen have noted that brown specks appear scattered throughout the mass. The question was accordingly raised whether these brown specks were due to discoloration by iodine which had been freed from combination with the potassium. If the discoloration were due to iodine then some iodine would also escape into the atmosphere and the remaining salt would contain less of it. The study to be reported in this paper concerns itself with the loss of iodine from iodized salt, the factors which influence its loss, and methods for preventing it.

## HISTORICAL

It is a common observation that solutions of potassium iodide become yellow or brown with age. The brown color of these solutions is due no doubt to the formation of free iodine resulting from the decomposition of potassium iodide. Concerning the mechanism or the dynamics of the change, little definite information is available. Exposure of the solution to sunlight apparently hastens the decomposition.

It is probable that the same factors which operate to liberate iodine from solutions of potassium iodide will operate in a similar way to produce free iodine in an iodized salt. Fellenberg (5)<sup>4</sup> finds that iodized salt in ordinary storage in a grocery store loses iodine. Since he found that the purer the salt the lower the loss of iodine, McClendon (13) suggests that the loss is probably due to the presence of nitrites or nitrates which oxidize the iodide to iodine, which then volatilizes into the air. Fellenberg (5) has determined the impurities likely to be present in salt. He found no correlation between the

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<sup>3</sup> Since the beginning of these experiments, unpublished results of the veterinary department indicate that stock salts carrying as low as 0.02 per cent of KI are effective in preventing goiter.

<sup>4</sup> Reference is made by number (*italic*) to "Literature cited," p. 183.

Mg, Ca, SO<sub>4</sub>, Fe, and Al content of the salt and the quantity of iodine lost during storage.

Since iodine should not escape so readily from an alkaline medium, this same investigator (5) prepared an alkaline iodized salt by the addition of 0.693 gm. of potassium carbonate per kilogram of the salt. In several instances this alkaline salt retained more of its iodine than ordinary iodized salt when the two salts were stored under comparable conditions. Fellenberg (5) also investigated the distribution of iodine stored in containers of such a nature that he could obtain upper, middle, and lower layers of the salt. He found that in every case the iodine content was lowest in the middle layer and greatest in the lowest layer. This phenomenon he attributed to the solubility of the potassium iodide in the moisture of the salt and its transportation to the surface by capillarity. At the surface it concentrates owing to the drying out of the salt. Fellenberg also states that slightly moist salt retains its iodine better than dry salt and, further, that iodized salt loses its iodine as it dries out.

Fellenberg (4) determined the hydrogen-ion concentration of solutions of stored salt from time to time. If iodine were lost from the salt then the remaining salt should be more alkaline, due probably to the formation of potassium bicarbonate. Fellenberg, however, observed no significant changes in hydrogen-ion concentration.

Exposure to air and light are likely to affect the rate of loss of iodine from materials containing it. Several workers have detected iodine in the atmosphere. Chatin (2) was able to classify regions as goiterous or nongoiterous according to the iodine content of the air. Gautier (11) found appreciable quantities of iodine in sea air, less in air at Paris, and none in mountain air. The iodine which this last worker detected was all present as organic iodine. Fellenberg (8) has determined the influence of exposure to light and air on the loss of iodine from materials containing it. He stored sea water from two sources for periods of two to seven weeks in darkness and in daylight, in still air and in moving air. Only the sea waters which were exposed to a current of air appeared to lose any iodine. Whether stored in darkness or in daylight made no difference in the quantity of iodine lost.

Another means by which iodine could be liberated from materials containing it is by the action of molds or bacteria. This means is of special significance in the case of stock salts which may be scattered or kept in blocks on the ground or fed in troughs which become contaminated with soil.

Fellenberg, Geilinger, and Schweizer (10) found that certain agencies in the soil act on potassium iodide to liberate iodine. When a strip of moist starch paper was suspended in a beaker containing soil with added potassium iodide, it turned blue in a short time, indicating the liberation of iodine. The evolution of iodine into the atmosphere occurred more readily in unfertilized than in fertilized soils. This might indicate that the nitrites or nitrates of the soil were not responsible for the reaction. It is quite likely, however, that iodine was also liberated in the fertilized soils but reacted with and was bound by the organic matter.

Concerning the mechanism by which the iodine was liberated, Fellenberg, Geilinger, and Schweizer (10) state that the reaction was due neither to bacteria nor enzymes but to inorganic catalysts.

The ferric ion seems the most likely catalyst. Corresponding soils containing ferrous iron liberated some iodine, but the reaction was less rapid. In the case of soils of low iron content the liberation of iodine was more rapid at the higher hydrogen-ion concentrations.

Fellenberg and Geilinger (9) also found that *Bacillus coli*, *Aspergillus niger*, and various molds bound free iodine when cultured in neutral media. These microorganisms were not specific or active, but purely passive in their ability to react with free iodine. The iodine merely combined with the protein of the microorganism as it would with any nonliving protein.

## EXPERIMENTAL

### ANALYTICAL PROCEDURE

The best method of determining minute quantities of iodine which may be present in a material are those developed by Fellenberg (4, 6, 7) and by McClendon (13). The quantity of potassium iodide used in iodized salt, however, is sufficiently large so that other methods were found more convenient. The iodized salts on the market in this country contain several hundredths of a per cent of potassium iodide, while the stock salt recommended by the veterinary department of the Montana Experiment Station contains 0.0625 per cent.

For the determination of such quantities of iodine as these, a modification of Kendall's method (12) was found most suitable. According to this method the iodide is oxidized to iodate by means of bromine. The excess bromine is then expelled by boiling, the last traces being removed by treatment with salicylic acid. After acidification with  $H_3PO_4$ , potassium iodide is added and the liberated iodine titrated with standard thiosulphate solution. The thiosulphate solution used was approximately 0.01 normal. One cubic centimeter of such a solution is equivalent to 0.0002116 gm. of original iodine. Throughout all the work the thiosulphate solution was checked against a standard solution of potassium biniodate. This method may be used when large quantities of sodium chloride are present. When relatively small quantities of bromides are present, however, low results are obtained.

### THE SALTS USED

The iodized salts were prepared from three types of commercial salts varying in size of particle. On the market these salts are known as table salt, hay salt, and stock salt. The salts were obtained from a salt manufacturer of Salt Lake City, Utah.

In the preliminary work attempts were made to prepare the iodized salts by adding potassium iodide to the salts in the form of a powder. Using this method it was found extremely difficult to obtain duplicate samples which would contain the same quantity of iodine. This was particularly true of the coarse stock salt. Since preliminary experiments indicated that some other method of preparing the iodized salt would have to be found, in order that uniform samples might be obtained, experiments were performed in which an aqueous solution of potassium iodide was added to the salt. It was thought that by this method the potassium iodide would be distrib-

uted more uniformly throughout the mass of salt and that on drying it would adhere to the salt particles. In any case the evaporation of the water would leave the potassium iodide more finely divided than by the previous method. By following this procedure it was possible to obtain more concordant results. It was accordingly adopted.

## RESULTS

In giving the results which were obtained when the various iodized salts were stored under different conditions, each of several variables in the conditions of storage will be considered separately. Among the factors which were thought important as affecting the rate of loss of iodine from iodized salt were the moisture content as determined by the humidity of the surrounding atmosphere, the acidity or alkalinity which might be superimposed upon the salt, and the method of preparation of the salt, as, for example, whether the potassium iodide were added as a powder or crystallized from the brine with the sodium chloride. Salts iodized with potassium iodate were also prepared and stored under the same conditions as salts iodized with potassium iodide.

The work was also extended to include observations on the loss of iodine from iodized salt exposed to direct sunlight and to rain. The effect of heat alone on the loss of iodine was also studied.

### STORAGE OF DRY IODIZED SALT IN PASTEBOARD CYLINDERS

Samples of hay salt and stock salt were prepared, containing approximately the quantity of iodine recommended by the veterinary department. The original salts were moistened with a solution of potassium iodide, and then before storing in the covered pasteboard cylinders the iodized salts were dried by spreading out in a thin layer at room temperature. Periodically for a year the iodine contents of the salts were determined. The results are given in Table 1. A study of this table shows that no significant changes in the iodine content of the salts had taken place. Hence, it is safe to conclude that iodized salts kept in this manner will retain most of their iodine for storage periods of considerable length. Fellenberg (5) reached similar conclusions in regard to iodized salt prepared for human consumption and kept on the shelves of a store until sold.

TABLE 1.—*The loss of iodine from iodized salts stored in pasteboard cylinders*

Date	Storage period	KI content	
		Hay salt	Stock salt
	Weeks	Per cent	Per cent
Oct. 5, 1925.....	0	0.0565	0.0610
Nov. 28, 1925.....	8	.0546	.0612
Feb. 4, 1926.....	17	.0553	.0597
May 7, 1926.....	31	.0570	.0603
June 30, 1926.....	38	.0563	.0610
Sept. 5, 1926.....	48	.0572	.0623
Oct. 18, 1926.....	54	.0571	.0615

## STORAGE OF IODIZED SALT UNDER SEMISHeltered CONDITIONS

Samples of iodized stock salt were stored in such a way as to allow free circulation of air through them. One sample in an open glass jar was placed in an open shed. Another sample in a burlap bag was stored in the loft of a barn. The iodine contents of these salts were determined after various intervals of time. The results obtained are given in Table 2. These data indicate that considerable losses of iodine occurred under these conditions of storage. The salt stored in the burlap bag appeared to lose iodine more rapidly than the one stored in the glass jar. Thus, after about 13 months the salt stored in the burlap bag had lost 14.4 per cent of its iodine, while that stored in the glass jar had lost only 6.1 per cent. Since air would circulate much more freely in the salt stored in the burlap bag and remove the free iodine more rapidly with consequent shifting of the conditions back to where more iodine would be liberated, it is possible that this may account for the greater loss of iodine from the salt subjected to more aeration.

TABLE 2.—*Loss of iodine from samples of iodized salt stored under semisheltered conditions*

Stored in open jar in shed					Stored in burlap bag in barn loft				
Date	Storage period	KI	Loss per 10-gram sample	Loss	Date	Storage period	KI	Loss per 10-gram sample	Loss
	Weeks	Per cent	Mgm.	Per cent		Weeks	Per cent	Mgm.	Per cent
Sept. 29, 1925.....	0	0.0605	0	0	Oct. 26, 1925.....	0	0.0629	0	0
Nov. 30, 1925.....	9	.0606	0	0	May 20, 1926.....	29	.0589	.40	6.8
Feb. 3, 1926.....	19	.0594	.11	1.8	July 20, 1926.....	38	.0572	.57	10.0
June 25, 1926.....	39	.0590	.15	2.5	Nov. 22, 1926.....	56	.0550	.79	14.4
Sept. 10, 1926.....	50	.0575	.30	5.2					
Nov. 14, 1926.....	60	.0570	.36	6.1					

## STORAGE OF IODIZED SALTS IN ATMOSPHERES OF DIFFERENT RELATIVE HUMIDITIES

Since iodized salts prepared on the range are likely to be stored under conditions differing in the relative humidity of the surrounding atmosphere, an experiment was devised to study this factor. The iodized salts were stored in desiccators in which the relative humidity was controlled by some substance or solution contained in the desiccator. The following relative humidities were maintained: 100, 50, 20, and 0 per cent. The 100 per cent relative humidity was maintained by having water in the desiccator, the 50 and 20 per cent by solutions of KOH, and the 0 per cent by solid  $\text{CaCl}_2$ . Potassium hydroxide was used for the intermediate relative humidities, as it would remove any free iodine from the atmosphere and hence would not allow the reaction by which iodine was liberated to stop or slow up because an equilibrium had been reached.

TABLE 3.—*Loss of iodine from iodized salts stored in atmospheres of different relative humidities*

Date	Storage period (weeks)	Percentage of KI at—			
		100 per cent relative humidity (over water)	50 per cent relative humidity (over KOH solution)	20 per cent relative humidity (over KOH solution)	0 per cent relative humidity (over $\text{CaCl}_2$ )
Oct. 2, 1925.....	0	0.0630	0.0615	0.0688	0.0650
Nov. 27, 1925.....	8	.0563	.0621	.0644	.0637
Feb. 3, 1926.....	18	.....	.0617	.0629	.0622
May 7, 1926.....	31	.0587	.0613	.0626	.0588
June 29, 1926.....	38	.....	.0618	.0620	.0630
Sept. 3, 1926.....	48	.....	.0615	.0620	.0635
Oct. 5, 1926.....	53	.....	.0610	.0593	.0622
Feb. 8, 1927.....	71	.0517	.....	.....	.....

The data which were obtained are given in Table 3. These data indicate that iodized salt stored over water loses considerable iodine. After May 9, 1926, it was impossible to sample this salt as it had taken up so much water. On February 8, 1927, the whole mass of salt was dissolved in water and the iodine content determined. The salt stored in an atmosphere of 100 per cent relative humidity lost nearly 20 per cent of its iodine during storage from October, 1925, to February, 1927. The salt stored over the solution of KOH of such concentration as to give 50 per cent relative humidity lost no iodine during storage, while the two salts stored in drier atmospheres, i. e., over the more concentrated solution of KOH and over  $\text{CaCl}_2$ , lost small quantities of iodine. Further observations concerning the loss of iodine from very dry iodized salts will be given later. Fellenberg obtained similar data when he stored dry and moist iodized salts.

#### STORAGE OF ACID AND ALKALINE IODIZED SALTS

It is known that when free iodine is introduced into an alkaline solution it reacts with the alkali to form the iodide. On the other hand, iodine is readily liberated from acid solutions of the iodide. Hence iodized salts rendered alkaline may retain their iodine better than neutral or iodized salts rendered acid. In order to test the validity of this idea, one iodized salt was rendered acid by the addition of 1 per cent of  $\text{KH}_2\text{PO}_4$  while another was rendered basic by the addition of 1 per cent of  $\text{NaHCO}_3$ . These salts were stored in a bell jar at ordinary humidity and the iodine content determined periodically. The results obtained are given in Table 4. The data in this table show that the salt rendered acid lost about one-third of its iodine during storage for approximately one year. The salt rendered alkaline not only did not lose but actually showed an increase in iodine content when stored for a year. Since the two salts were stored under the same bell jar, the basic salt apparently absorbed a small quantity of the iodine liberated from the acid salt. That iodine was liberated from the acid salt and not from the basic salt was shown qualitatively. A piece of paper was placed in each salt. The paper placed in the acid salt became dark brown, while that in the basic salt showed no discoloration.

TABLE 4.—*The influence of added acid or alkaline salts on the loss of iodine from iodized salts*

Date	Storage period	KI content	
		Salt containing 1 per cent $\text{KH}_2\text{PO}_4$	Salt containing 1 per cent $\text{NaHCO}_3$
	Weeks	Per cent	Per cent
Oct. 2, 1925.....	0	0.0610	0.0622
Nov. 28, 1925.....	8	.0622	.0631
Feb. 4, 1926.....	18	.0509	.0630
May 7, 1926.....	31	.0468	.0640
June 28, 1926.....	38	.0451	.0645
Sept. 3, 1926.....	48	.0428	.0648
Oct. 13, 1926.....	54	.0410	.0649

The effect of the alkalinity in preventing loss of iodine from iodized salt is not in entire agreement with certain work of Fellenberg. He found that the addition of 0.693 gm. of  $\text{K}_2\text{CO}_3$  to a kilogram of salt did not always prevent the loss of iodine, although in some cases it did. Fellenberg did not work with an acid salt. It is possible that a slight loss of iodine from the alkaline salt would have been found in the writers' experiments also if the atmosphere had not been so rich in iodine liberated from the salt which had been rendered acid. Data will be presented later, however, to show that the addition of  $\text{NaHCO}_3$  distinctly reduces the loss of iodine from iodized salt.

#### POTASSIUM IODIDE LEACHED FROM IODIZED SALT BY RAINS

In order to study the effect of exposure to climatic conditions, particularly rain, on the loss of potassium iodide from salts containing it, a large deep funnel containing 2,240 gm. of the salt was set up out of doors. A bottle was placed under the funnel in order to catch the solution which would seep through the salt. It was thought that almost all the potassium iodide which was present in the salt would be removed by the first rain (on account of the great solubility of KI in water), and thereafter the residual salt would be practically iodine free. Such was not the case, however, as the data in Table 5 indicates. After several rains resulting in the collection of 667 gm. of leachings the salt still contained more than 20 per cent of its original potassium iodide. One would expect, moreover, to obtain more iodine in the first rains than in later rains. The rate of rainfall, however, appears to regulate this. The first rains were very hard, while those which came later were slower.

The reason that more potassium iodide is not removed is probably because the rain washes channels down through the salt and the channels once formed allow most of the water to pass through. The remainder of the salt was protected by air which was unable to escape through the wetted salt surrounding it. Hence the water does not come in contact with the potassium iodide in the body of the salt but only with that along the channel. The formation of these channels in the salt layer was checked up in the laboratory. Salt was placed in long tubes and water from a burette allowed to drip slowly on the salt. The effect of the water as it seeped through the tube was then noted.

TABLE 5.—Potassium iodide leached from iodized salt by rain

Date	Rainfall	Weight of leachings	Total solids in leachings	KI extracted	Percentage of original KI extracted
		Inches	Grams	Per cent	
July 29, 1925 <sup>a</sup> .....	0.31	114.0	14.0	109.0	8.5
Do.....		37.9	26.1	62.0	4.8
Aug. 3, 1925.....	0.03				
Aug. 13, 1925.....	0.01				
Aug. 14, 1925 <sup>a</sup> .....	0.64	109.2	20.7	214.5	16.7
Aug. 15, 1925.....	0.01	196.0		292.5	22.7
Aug. 20, 1925.....	0.01				
Aug. 27, 1925.....	0.38				
Aug. 28, 1925.....	0.21	210.7	25.0	321.0	25.0
Total.....	1.60	667.8		999.0	77.7

<sup>a</sup> Collected in two portions.

At the end of the experiment the salt remaining in the funnel was dissolved in water, and the potassium iodide in an aliquot of solution was determined. The leachings contained 77.7 per cent and the residual salt 22.4 per cent of the original potassium iodide, respectively, making a total of 100.1 per cent. This indicates that all of the iodine is accounted for in the leachings and in the residue and that very little could have been liberated by sunlight or other climatic factors. The experiment was, however, too crude to enable one to make accurate observations concerning the effect of sunlight in liberating iodine from iodized salt.

#### THE EFFECT OF METHOD OF CRYSTALLIZATION ON THE LOSS OF IODINE FROM IODIZED SALT

In most of the study which has been recorded the iodized salts were prepared by moistening the sodium chloride with a solution containing the proper quantity of potassium iodide. It was thought that it might be possible to prepare iodized salt in which the potassium iodide would be either occluded or held in solid solution within the crystals of sodium chloride. If such were possible the rate of loss of iodine should be slower than when the potassium iodide is merely coated on the outside of the sodium chloride crystals, as is probably the case when the salts are iodized by adding a solution of potassium iodide. In following out this idea an iodized salt was dissolved in water and, by evaporation of the water, it was recrystallized. The salt was then dried and stored, in one instance, in a pasteboard carton and, in another instance, in a canvas bag. The same iodized salt but not recrystallized was stored in an open glass jar. The iodine content of the three salts was determined periodically. The results are given in Table 6. A study of the data in this table indicates that the recrystallized salt stored in the pasteboard carton lost no iodine during storage for a year. This is in agreement with the data in Table 1 in which several salts were stored in the same manner. A sample of the same recrystallized salt stored in a canvas bag lost about the same quantity of iodine (a slightly greater percentage) as did the iodized salt not recrystallized. Hence, it would appear that no advantage attends recrystallizing the salt in this manner.

TABLE 6.—*Comparison of losses of iodine from ordinary iodized salt and iodized salt recrystallized*

Recrystallized iodized salt stored in pasteboard carton			Recrystallized iodized salt stored in canvas bag			Iodized salt (not recrystallized) stored in open glass jar		
Date	Storage period	KI content	Date	Storage period	KI content	Date	Storage period	KI content
	<i>Weeks</i>	<i>Per cent</i>		<i>Weeks</i>	<i>Per cent</i>		<i>Weeks</i>	<i>Per cent</i>
Oct. 19, 1925.....	0	0.0794	Oct 19, 1925.....	0	0.0798	Aug. 18, 1925.....	0	0.1205
Jan. 19, 1926.....	13	.0794	Dec. 21, 1925.....	9	.0762	Oct 19, 1925.....	9	.1150
May 7, 1926.....	29	.0797	Feb. 3, 1926.....	15	.0751	Dec. 21, 1925.....	18	.1137
June 30, 1926.....	36	.0797	May 1, 1926.....	28	.0743	May 8, 1926.....	38	.1138
Sept. 7, 1926.....	46	.0792	June 30, 1926.....	36	.0720	June 30, 1926.....	45	.1130
Oct. 18, 1926.....	52	.0794	Sept. 3, 1926.....	46	.0720	Sept. 5, 1926.....	55	.1132
			Nov. 22, 1926.....	57	.0705	Nov. 1, 1926.....	63	.1125

In order to determine further whether mixed crystals of potassium iodide and sodium chloride were formed, the following experiment was performed: Seventy grams of iodized salt was dissolved in just sufficient water to dissolve it, and about half the water evaporated. The salt which separated out was then placed in a Büchner funnel and sucked dry. Then enough water was added so that a pasty mush was formed. This mush was stirred for a few minutes and finally the water was drawn off by suction. This process was repeated. If a solid solution of potassium iodide in sodium chloride had been formed during the recrystallization then the mother liquor as well as the crystals remaining on the filter should contain considerable quantities of potassium iodide. If a solid solution had not been formed then the potassium iodide should all be dissolved in the mother liquor due to its relatively greater solubility. The salt remaining on the filter was found to contain practically no potassium iodide, hence it may be concluded that mixed crystals had not been formed. Since the recrystallized salt used in the storage experiment had been prepared in this manner, there is no reason to expect that it would retain its iodine to any greater degree than salt iodized in the ordinary way. Since sodium chloride and potassium iodide are of similar crystal structure it might be possible to prepare mixed crystals containing the two salts if proper conditions were maintained during crystallization. The preparation of solid solutions of several of the alkali halides is recorded in the literature. Such mixed crystals of potassium iodide and sodium chloride would probably contain much more iodine than is present in iodized salt, and would have to be used for iodizing salt. There is the possibility that salts iodized with mixed crystals of potassium iodide and sodium chloride would lose their iodine at a slower rate than salt iodized with potassium iodide. In view of the relatively small losses of iodine from salts stored under even only moderately favorable conditions this possibility did not seem worthy of investigation.

#### THE EFFECT OF SUNLIGHT ON THE LOSS OF IODINE FROM IODIZED SALT

Since in the feeding of iodized salts to stock the salts are likely to be exposed to sunlight for various periods of time, and since it is known that sunlight hastens the decomposition of potassium iodide,

a study was made of the effect of sunlight on the rate of loss of iodine from iodized salts. The salts were stored in open crystallizing dishes on the sill of a south window and determinations of the iodine content were made periodically. Four salts were used in this study, (1) a salt iodized with finely divided  $KIO_3$ , (2) a salt iodized with KI solution, (3) and (4) two iodized salts which had been recrystallized according to the method used in the preceding section. The results obtained are given in Table 7. The most significant conclusion that can be drawn from these data is that salts iodized with  $KIO_3$  retain their iodine when exposed to sunlight, while salts iodized with KI lose considerable portions of it. Thus, the salt which originally contained 0.0620 per cent of KI contained only 0.0165 per cent at the end of 63 weeks. The recrystallized salts show no advantage over ordinary iodized salts in regard to retaining their iodine when exposed to sunlight. Salt (4) was stored in a crystallizing dish of  $4\frac{3}{8}$  inches diameter while the other salts were stored in dishes of  $7\frac{1}{2}$  inches diameter. Hence, a smaller surface of the salt was exposed to the light. This probably accounts for the smaller loss of iodine in this case.

TABLE 7.—*Loss of iodine from several iodized salts stored on the sill of a south window. Salt (1) was iodized with  $KIO_3$  powder; salt (2) with KI solution; salts (3) and (4) were iodized with KI and recrystallized*

Date	Storage period	KI content			
		Salt 1	Salt 2	Salt 3	Salt 4
	Weeks	Per cent	Per cent	Per cent	Per cent
July 30, 1925.....	0	0.0630	0.0620	0.0528	.....
Oct. 5, 1925.....	10	.0628	.0495	.0270	0.0780
Nov. 28, 1925.....	17	.0620	.0399	.0179	.0745
Feb. 4, 1926.....	27	.0613	.0355	.0173	.0729
May 7, 1926.....	40	.0627	.0299	.0072	.0694
June 28, 1926.....	48	.0630	.0280	.0065	.0663
Sept. 3, 1926.....	57	.0633	.0250	.0049	.0621
Oct. 13, 1926.....	63	.0636	.0165	.0040	.0588

Since the surface exposed to the light appeared to be a factor in determining the quantity of iodine lost from an iodized salt, this factor was subjected to further study. Ten gram samples of iodized salt were weighed out on watch glasses and some of the watch glasses containing the salt were placed in dark cupboards, others were placed on the roof in direct sunlight, and still others were placed on the roof in the sunlight but covered with other watch glasses. The data which were obtained are given in Table 8. The losses in iodine which occurred are relatively large. Thus, exposure of the salt for two days resulted in a loss of 40 per cent of the iodine. When the watch glass containing the salt was covered the loss was less, or only 14.9 per cent. In the experiment in which one day's exposure was allowed the sky was somewhat cloudy. This may account for its lower loss in proportion to the loss for two days. The losses in all cases, however, are relatively large.

TABLE 8.—*Effect of exposure to sunlight on the rate of loss of iodine from iodized salt*

Treatment	Exposure of a salt for 1 day	Exposure of another salt for 2 days
Control (stored in dark cupboard), per cent KI.....	0.1662	0.0530
Exposed to direct sunlight, per cent KI.....	.1505	.0316
Difference.....	.0157	.0214
Loss, per cent.....	9.4500	40.300
Control (stored in dark cupboard), per cent KI.....	.1662	.0530
Exposed to sunlight but kept under glass, per cent KI.....	.1557	.0451
Difference.....	.0105	.0079
Loss, per cent.....	6.4900	14.900

## EXPOSURE OF IODIZED SALTS TO VARIOUS CONDITIONS OF TEMPERATURE AND LIGHT

In the data which have been recorded thus far it has developed that losses of iodine were prevented when the salts were iodized with  $\text{KIO}_3$  and when salts iodized in the ordinary way had added to them 1 per cent of  $\text{NaHCO}_3$ . It would have been most desirable to expose such salts to direct sunlight in order to determine their efficiency in retaining iodine under such a condition. Such experimentation as was carried out in the preceding section was, however, very difficult, as in many cases the watch glasses containing the salts were upset by wind or rain and the whole experiment had to be repeated. In the experiments to be described in this section, artificial conditions of heat and light were therefore set up with the idea of paralleling as closely as possible natural conditions of exposure to sunlight. An attempt was also made to determine what wave lengths of light were responsible for the decomposition of potassium iodide. The results which were obtained, when several salts were exposed to varying degrees of heat and light, are given in Table 9. Salts A, B, and C were iodized with potassium iodide, C being the neutral salt, while A contained 1 per cent of added  $\text{NaHCO}_3$  and B 1 per cent of  $\text{KH}_2\text{PO}_4$ . Salt D was a neutral salt iodized with  $\text{KIO}_3$ . Ten gram portions of each of the salts were weighed out into small evaporating dishes and exposed to the different degrees of light or heat.

TABLE 9.—*Exposure of several iodized salts to various conditions of light and heat. Salt A contained 1 per cent  $\text{NaHCO}_3$ ; salt B, 1 per cent of  $\text{KH}_2\text{PO}_4$ ; salt C was the neutral salt; and salt D was a neutral salt iodized with  $\text{KIO}_3$* 

Treatment	Percentage of KI in—			
	Salt A	Salt B	Salt C	Salt D
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
None.....	0.0568	0.0561	0.0569	0.0600
Exposure to ultra-violet light for 10 minutes.....	.0562	.0550	.0567	.0595
Exposure to infra-red light for 30 minutes.....	.0568	.0551	.0568	.0595
Exposure to electric light for 70 hours.....	.0551	.0443	.0520	.....
Exposure to electric light for 100 hours.....	.0555	.0405	.0511	.0603
Exposure to electric light for 167 hours.....	.0530	.0387	.0497	.....
Exposure to heat on hot plate at about 80° C. for 70 hours.....	.0543	.0424	.0395	.0590
Exposure to heat on hot plate at about 80° C. for 77 hours.....	.0556	.0416	.0394	.0602
Exposure to electric light for 167 hours, last 67 hours on hot plate at about 80° C.....	.0545	.0346	.0351	.....

In one instance the salts were exposed for 10 minutes to ultra-violet light generated by an apparatus used by a local physician. The salts were placed about 4 feet from the source of the light. A study of the data in Table 9 indicates that none of the salts lost significantly great quantities of iodine. The salt with the acid reaction, however, showed the greatest loss. It is likely that the time of exposure was not long enough to allow the decomposition to proceed very far. The apparatus was such, however, that the light could not be generated for longer periods of time without injury to the apparatus.

When portions of the same salts were exposed to infra-red rays (also produced by a physician's apparatus) for a period of 30 minutes at a distance of 3 feet from the source of light, only very slight losses of iodine occurred. It is significant, however, that again the salt rendered acid with  $\text{KH}_2\text{PO}_4$  lost the greatest quantity of iodine. It is also probable in this case that exposure to the light for longer periods of time would have given more significant results, but again this was not possible.

Portions of salts A, B, C, and D were next exposed to the light from a 750-watt electric light bulb, the dishes containing the salts being placed 10 inches from the filament. One series was exposed for 70 hours, another for 100 hours, and a third for 167 hours. At the end of these periods of exposure the iodine contents were determined. The data show that the salt containing the iodate lost none of its iodine; salt A, rendered alkaline with  $\text{NaHCO}_3$ , lost very little; while the neutral and acid salts lost considerable quantities which increased as the time of exposure to the light was increased. Also the acid salt lost appreciably more than the neutral salt. This result might be expected as the instability of the iodides in acid solution is well known.

The effect of heat alone on the loss of iodine from the same series of salts was next investigated. The dishes containing the salts were placed on a hot plate with the heat turned "low." The temperature attained by the salt was found to be about  $80^\circ\text{C}$ . A study of the data in Table 9 shows that the salt iodized with iodate again lost none of its iodine and that the loss from the salt rendered alkaline was relatively slight. Salts B and C again lost considerable quantities of their iodine. It is interesting to note that in this experiment the neutral salt lost more of its iodine than the one rendered acid with  $\text{KH}_2\text{PO}_4$ . This behavior occurred in two instances when the salts were exposed to heat alone; hence it is not likely that it is due to experimental errors. The explanation for the phenomenon is at present unknown.

Salts A, B, and C were next exposed to heat and light simultaneously. Salt D was omitted from this experiment as it had already been shown to be stable when exposed to either heat or light alone. Salt A again showed itself to be very stable. It lost only slightly more iodine than when exposed to light alone for the same time. In every case the losses of iodine from the salt rendered alkaline with  $\text{NaHCO}_3$  are slight. Salts B and C lost about 60 per cent of their iodine. The losses in the case of these two salts were greater than when they were exposed to light alone for the same period of time. Thus the iodine content of the neutral salt decreased from 0.0569 per cent to 0.0497 per cent when exposed to light alone, while when

kept at 80° C. for the last 67 hours of the 167-hour exposure to light it decreased further to 0.0351 per cent. The acid salt showed similar decreases from 0.0561 per cent to 0.0387 per cent and to 0.0346 per cent, respectively. From these data it is evident that both light and heat are factors affecting the rate of loss of iodine from iodized salt.

The experiments of Fellenberg (5) as well as some of those of the present writers, showed that iodized salts which were kept perfectly dry lost more iodine than moist salts. The factor of dryness and the effect of heat alone on the salt may be linked together in some way, as the salt kept at 80° C. on the hot plate was certainly dry.

Concerning the mechanism of the reactions which take place when iodine is liberated in solutions of potassium iodide or from iodized salt, little definite information is available. Since iodine is set free, the solution or the salt, as the case may be, should have an alkaline reaction due to an excess of base. To test out this idea, a solution of KI which had stood in the laboratory for several years and had become a dark brown was decolorized by the addition of finely divided silver. The silver reacted with the free iodine to form silver iodide, and when filtered the solution became water clear. The resultant solution was red to phenolphthalein and when titrated with standard acid it was found to be 0.013 normal in its alkalinity; with methyl orange the alkalinity was 0.016. Why such an alkaline solution does not cause the combination of iodine and alkali to reform the iodide is not known unless the iodine exists in some complex combination, which is probably the case.

Fellenberg (4) attempted to measure the decomposition of iodized salt by determining the hydrogen-ion concentration of a solution of the original salt and then that of the salt after storage. An increase in alkalinity would indicate loss of iodine. He found that solutions of the salts prepared before and after storage showed no significant difference in hydrogen-ion concentration. The quantity of KI in the iodized salts which he used was, however, so low<sup>5</sup> as to render difficult the determination of any slight changes which may have taken place. In the writers' work it was found that whenever any appreciable loss of iodine occurred there was also a resulting change in hydrogen-ion concentration. Thus, at the end of the storage period when drops of indicators were placed upon small portions of the salt, the salt stored in an atmosphere of 100 per cent relative humidity (Table 3), which had lost a considerable quantity of iodine, was shown to be more alkaline than the same salt which was stored in an atmosphere of 50 per cent relative humidity.

This same fact was shown in another set of experiments. The hydrogen-ion concentrations of the series of salts listed in Table 9 as being exposed to the light of the 750-watt electric light bulb were determined before and after exposure to the light. The results are given in Table 10. A comparison of the values for iodine lost as given in Table 9 and changes in  $P_H$  as given in Table 10 shows the relation which exists. The salts which lost the most iodine show the greatest change in the hydrogen-ion concentration of their solutions. With the hydrogen electrode it was impossible to determine the hydrogen-ion concentration of solutions containing the iodate.

<sup>5</sup> The salts with which Fellenberg worked contained five parts of KI per million parts of the salt. This quantity of KI was in accordance with the recommendations of the Swiss Goiter Commission.

Colorimetric determinations indicated, however, that the acidity of the salt iodized with  $\text{KIO}_3$  had not been changed by exposure to the light.

TABLE 10.—*The hydrogen-ion concentration of solutions of several iodized salts. Salt A contained 1 per cent of  $\text{NaHCO}_3$ ; salt B, 1 per cent of  $\text{KH}_2\text{PO}_4$ ; salt C was the neutral salt; salt D was a neutral salt iodized with  $\text{KIO}_3$*

Salt	Hydrogen-ion concentration in terms of $\text{P}_\text{H}$ <sup>a</sup>		Salt	Hydrogen-ion concentration in terms of $\text{P}_\text{H}$ <sup>a</sup>	
	Original	Final		Original	Final
A.....	7.13	7.25	C.....	6.54	7.02
B.....	2.62	3.50	D.....		

<sup>a</sup> The hydrogen-ion concentrations were determined before and after the salts had been exposed to electric light for 100 hours.

#### RELATION BETWEEN THE QUANTITY OF IODINE PRESENT IN AN IODIZED SALT AND THE QUANTITY LOST

In order to determine whether a certain proportion of iodine was lost from an iodized salt or whether an absolute quantity of it was lost, two salts were prepared containing widely different percentages of iodine. These salts were stored and their iodine content determined periodically. The results which were obtained are given in Table 11. The data in this table indicate that a certain percentage of the iodine is lost and not an absolute quantity of it. Thus, during the same storage periods the sample containing originally 0.1205 per cent of KI lost 0.08 mgm. of KI per gram of salt, while that containing originally 0.0288 per cent lost 0.02 mgm. of iodide per gram of salt. Of KI lost, the sample containing 0.1205 per cent of KI lost 6.65 per cent, while that containing 0.0288 per cent lost 6.95 per cent.

TABLE 11.—*Relation of quantity of KI present in an iodized salt to that lost during storage*

Date	Iodized salt of high KI content		Iodized salt of low KI content	
	KI present	KI lost	KI present	KI lost
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Aug. 18, 1925.....	0.1205		0.0288	
Oct. 19, 1925.....	.1150	4.57	.0282	2.08
Dec. 21, 1925.....	.1137	5.65	.0280	2.78
May 8, 1926.....	.1138	5.57	.0281	2.43
June 30, 1926.....	.1130	6.23	.0274	4.85
Sept. 5, 1926.....	.1132	6.06	.0275	4.51
Nov. 1, 1926.....	.1125	6.65	.0268	6.95

Table 12 gives results from which similar conclusions may be drawn. In this case two series of salts containing different quantities of iodine were exposed to electric light for 100 hours. The quantity of iodine lost apparently depends on the quantity present.

TABLE 12.—*Relation of quantity of KI present in an iodized salt to that lost by exposure to electric light for 100 hours. Salt A contained 1 per cent of  $\text{NaHCO}_3$ ; salt B, 1 per cent of  $\text{KH}_2\text{PO}_4$ ; salt C was the neutral salt*

KI content	Salts of high KI content			Salts of low KI content		
	Salt A	Salt B	Salt C	Salt A	Salt B	Salt C
Initial KI content, per cent.....	0.0568	0.0561	0.0569	0.0114	0.0112	0.0114
Final KI content, per cent.....	.0555	.0495	.0511	.0109	.0084	.0095
Difference.....	.0013	.0156	.0058	.0005	.0028	.0019
Per cent loss.....	2.29	27.80	10.20	* 7.00	25.00	16.7

\* A single determination, others in duplicate.

## DISCUSSION

From the data which have been given it is evident that iodized salts show considerable variation in the degree to which they retain their iodine when stored for extended periods of time. It appears that when air is able to circulate freely over the salt or through it the loss of iodine is greater than when circulation of air is prevented. On this basis coarse-grained iodized salts should lose their iodine more rapidly than fine-grained salts. In several instances this has been found to be true. The data indicate quite clearly that when circulation of air is prevented, loss of iodine for all practical purposes does not occur even when the salt is stored for periods longer than a year, provided the salts are not too damp and provided they are not exposed to light of too great intensity. Salts may be stored in atmospheres up to at least 50 per cent relative humidity without serious loss of iodine. In fact, iodized salts stored in atmospheres of 50 per cent humidity appear to retain their iodine more effectively than salts stored in drier atmosphere.

Exposure of ordinary iodized salts to sunlight results in setting free very considerable quantities of the iodine which is present. In one instance 40 per cent of the iodine of an iodized salt was lost when a thin layer of the salt was exposed to direct sunlight for two days. This loss of iodine by the action of light may be reduced either by iodizing the salt with  $\text{KIO}_3$  or by rendering it alkaline with 1 per cent of  $\text{NaHCO}_3$ . It is possible that a smaller quantity of  $\text{NaHCO}_3$  might have been sufficient, but investigations with the addition of other quantities were not made in this study. Concerning the advisability of replacing KI with  $\text{KIO}_3$  there may be a question. Crespolani (3) states that small quantities of  $\text{KIO}_3$  are changed in the intestine to KI, and hence utilized in the same way as KI. The principal agency in the reduction which takes place is thought to be peptones in acid solution. He administered as much as 2 to 3 gm. at a dose. When such large doses were administered, however, some  $\text{KIO}_3$  was excreted unchanged in the urine. Mellor (14, p. 598), on the other hand, states that care must be exercised in the choice of KI for medicinal purposes as it may contain some  $\text{KIO}_3$ , which, according to him, is a poison. It is not believed, however, that the small quantity of  $\text{KIO}_3$  which would be taken internally in iodized salt would have any ill effects on the organism. In this regard McClendon in a private communication states that  $\text{KIO}_3$  may be administered orally to human beings to the extent of  $\frac{1}{4}$  gm. per

kilogram body weight. He advises against its injection into the system. Crespolani (3) also states that intravenous, intramuscular, or subcutaneous injection of iodates does not result in their reduction to iodides. From this he concludes, as has already been stated, that the reduction occurs in the alimentary canal. The use of  $\text{KIO}_3$  to replace  $\text{KI}$  in iodized salt must, however, be a subject for further research.

According to Chapline and Talbot (1), the best way in which to feed salt to sheep is at the camp each night. Only what will be eaten is fed each time; hence there is no danger of losing iodine by exposure to sunlight. When, however, the salt is kept in a trough in the open sunlight for days or weeks at a time, considerable quantities of iodine will be lost before the salt has been eaten. The same is true of blocks or cubes of salt kept on the range. The iodine in the surface of the cubes may entirely disappear between the times when stock remove the outer surface. It would appear, therefore, that the use of iodized salt in blocks would not be a desirable practice.

At the beginning of the work it was reported that iodized salts containing brown spots were found, but no such spots have come under the observation of the writers. Van de Vorst (15) states that brown spots developed in potassium iodide due to the growth of a certain fungus. Such fungus growth might have caused the brown spots reported by the veterinary department.

### CONCLUSIONS

Iodized salts stored in atmospheres of relative humidities of 50 per cent lose smaller quantities of their iodine than salts stored under similar conditions at other humidities.

Iodized salts rendered alkaline by the addition of  $\text{NaHCO}_3$  lose practically none of their iodine during storage, while neutral salts or salts rendered acid lose appreciable quantities.

Salts iodized with  $\text{KIO}_3$  lose none of their iodine when stored for extended periods.

Exposure of iodized salts to sunlight effects a loss of iodine from neutral or acid salts, only a slight loss from salt rendered alkaline, and practically no loss from salts iodized with  $\text{KIO}_3$ .

Exposure to heat alone effects losses of iodine from acid, neutral, and alkaline iodized salts and from salt iodized with  $\text{KIO}_3$  in precisely the same order as did exposure to sunlight.

Exposure to light and heat simultaneously effects greater losses of iodine from salts of neutral or acid reaction than exposure to light alone or heat alone. In the case of iodized salts of alkaline reaction the losses of iodine are again insignificant or negligible, even though the salts are exposed to light and heat simultaneously.

The quantity of iodine liberated from a neutral salt iodized with  $\text{KI}$  appears to depend on the quantity of  $\text{KI}$  present.

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# THE CURRENT MINERAL NUTRIENT CONTENT OF THE PLANT SOLUTION AS A POSSIBLE MEANS OF CHEMICAL CONTROL OF OPTIMUM FERTILIZATION<sup>1</sup>

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## INTRODUCTION

During 1926 an attempt was made to estimate the current nutrient needs of various crops growing under various levels of fertilization. In order to secure such information determinations were made at intervals during the growing season of the nitrate nitrogen, phosphate phosphorus, and potassium contents of the plant solutions obtained from field crops growing in appropriate situations. In addition to these determinations, total analyses of the fertilizer ingredients were made on samples of tissue collected at the end of the growing season or, in a few cases, at various times during the season. In this paper the results obtained are given and the possibility of using the current levels of nutrient elements as bases for chemical control of fertilization is discussed.

## ANALYTICAL METHODS AND CULTURAL CONDITIONS

The analytical methods employed with the plant solutions were those discussed by Gilbert in earlier papers.<sup>2, 3</sup>

The methods of analyses for the constituents of plant tissue used by the Association of Official Agricultural Chemists<sup>4</sup> were used for the total analyses in this study, with the exception that in the preparation of the samples for the determinations of  $P_2O_5$  and  $K_2O$  a wet digestion with sulphuric and nitric acids was employed.

The fertilizer ingredients applied per acre are shown in Table 1. In each experiment, with the exception of the one in which nitrogen was used on the phosphate area, one fertilizer ingredient was definitely made suboptimal, past experience indicating that the quantities of the other fertilizers used were sufficient for the production of a normal crop. On the phosphate area the  $P_2O_5$  was applied in acid phosphate; on the potash area the  $K_2O$  was applied in high-grade sulphate of potash; and on the market-garden area the nitrogen was applied in nitrate of soda, sulphate of ammonia, and tankage.

The growing season of 1926 seems to have been quite free from serious climatic growth-inhibiting conditions. Growth measurements on several crops and a chart of soil and air temperatures, accompanied by rainfall, are given in an earlier paper.<sup>5</sup> It was

<sup>1</sup> Received for publication May 2, 1927; issued August, 1927. Contribution No. 347 of the Rhode Island Agricultural Experiment Station.

<sup>2</sup> GILBERT, B. E. THE ADAPTATION OF CERTAIN COLORIMETRIC METHODS TO THE ESTIMATION OF NITRATES, PHOSPHATES, AND POTASSIUM IN PLANT SOLUTIONS. *Plant Physiol.* 1:191-199. 1926.

<sup>3</sup> GILBERT, B. E., McLEAN, F. T., and ADAMS, W. L. THE CURRENT MINERAL NUTRIENT CONTENT OF THE PLANT SOLUTION AS AN INDEX OF METABOLIC LIMITING CONDITIONS. *Plant Physiol.* (In press.)

<sup>4</sup> ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. OFFICIAL AND TENTATIVE METHODS OF ANALYSIS. COMPILED BY THE COMMITTEE ON EDITING METHODS OF ANALYSIS. REVISED TO JULY 1, 1924. Ed. 2, 535 p., illus. Washington, D. C. 1925.

<sup>5</sup> GILBERT, B. E., McLEAN, F. T., and ADAMS, W. L. *Op. cit.*

there reported that "the only noticeable depression in growth increments which may be attributed to temporary unfavorable conditions for growth, was during the period from July 15 to July 29." This was accompanied by a low rainfall and a high temperature period. The following statements were also made: "The moisture content of the soil was not critically low at any time during the summer. It fluctuated between 20 per cent and 35 per cent of the dry weight of the soil. The calculated wilting coefficient of the soil was about 12 per cent and the usually accepted optimum moisture content of the soil for plant growth was approximately 18 to 22 per cent."<sup>6</sup>

The highest yields of different varieties of crops investigated under different fertilizer treatments are given in Table 2. Subsequent tables give the relative yields for all plots based on the yields in Table 2, which are taken as 100. Due to the small areas planted and to the small number of plant individuals, the yields on the phosphate experiment were not expected to be of significance, the crops having been provided only for analyses.

TABLE 1.—Fertilizer ingredients applied per acre to different plots

Plot	N	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O	Plot	N	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O
	Pounds	Pounds	Pounds		Pounds	Pounds	Pounds
55N.....	<sup>a</sup> 55+40	30	135	116 MG (spring).....	0	200	30
65N.....	<sup>a</sup> 55+40	130	135	118 MG (spring).....	100	200	100
65S.....	<sup>a</sup> 80+60	150	135	119 MG (spring).....	<sup>d</sup> 0+20	200	100
116.....	<sup>b</sup> 80+40	210	0	115 MG (fall).....	<sup>d</sup> 70+40	120	90
117.....	<sup>b</sup> 80+40	210	50	116 MG (fall).....	<sup>d</sup> 0+53	120	90
119.....	<sup>b</sup> 80+40	210	150	118 MG (fall).....	<sup>d</sup> 70+20	120	90
115 MG (spring).....	60	200	30	119 MG (fall).....	<sup>d</sup> 35+10	120	90

<sup>a</sup> Side dressings of NaNO<sub>3</sub> applied.

<sup>b</sup> Side dressings of cyanamid applied.

<sup>c</sup> These spring applications supplemented 16 tons of stable manure.

<sup>d</sup> Side dressings of  $\frac{1}{2}$  NaNO<sub>3</sub> and  $\frac{1}{2}$  (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> applied.

TABLE 2.—Crops investigated and highest yields obtained from different varieties

Crop	Area	Number of rows	Variety	Kind of tissue examined	Highest yield
Cabbage.....	Phosphate <sup>a</sup> .....	1	Enkhuizen.....	Trimmed heads.....	<i>Pounds per row</i> 208
Do.....		1	Ballhead.....	do.....	129
Carrots.....		1	Danvers' Half Long.....	Roots.....	184
Turnips.....		1	Macomber Rutabaga.....	do.....	147
Buckwheat.....		2	Japanese.....	Green plants.....	40
Corn.....	Potash <sup>b</sup> .....	5	R. I. White Cap.....	Grain and cob.....	<i>Cwt. per acre</i> 67
Turnips.....		8	Macomber.....	Roots.....	366
Parsnips.....		6	Champion Hollow Crown.....	do.....	400
Potatoes.....		5	Rural Russet.....	Tubers.....	251
Tomatoes.....		6	Livingston's Globe.....	Fruit.....	482
Cabbage.....	Market garden <sup>c</sup> .....	8	Ballhead.....	Trimmed heads.....	355
Lettuce.....		.....	Black Seeded Tennis Ball.....	do.....	260
Celery.....		.....	Golden Plume.....	Untrimmed heads.....	339
Cabbage.....		.....	Golden Acre.....	Trimmed heads.....	467
Beets.....		.....	Early Wonder and Crosby's Egyptian (mixed). .....	Roots.....	224

<sup>a</sup> Rows were 96 feet in length.

<sup>b</sup> Rows were 30 feet in length.

<sup>c</sup> Crops covered whole plots (21 by 69.14 feet).

<sup>d</sup> GILBERT, B. E., McLEAN, F. T., and ADAMS, W. L. Op. cit.

## CURRENT NITRATE-NITROGEN CONTENT OF PLANTS STUDIED

Four market-garden crops were selected for study. Of these, two were planted in the spring and two in the fall. Lettuce seed was planted April 16 and cabbage plants were set April 22. After these two crops were harvested beets were sown July 23 and celery plants were set July 19, on the respective areas.

In Table 3 the current nitrate nitrogen and the total nitrogen in tissue at time of harvest are shown, as well as the relative yields, and the quantity of nitrogen applied in chemical fertilizers. Each crop responded to the extra nitrogen application, and this extra quantity was reflected consistently in the plant solution throughout the season, the plants on the higher nitrogen plot having higher nitrate nitrogen in solution content. The total nitrogen results also correlate in general with these. The magnitude of the nitrate-nitrogen content is doubtless affected by side dressings of nitrogen made during the season. On May 27 an additional 20 pounds of nitrogen was applied to cabbages on plot 119. Celery on plots 115 and 116 received an extra application September 7, and beets on plots 118 and 119 were given 20 and 10 pounds, respectively, September 9. These applications are all reflected in the solution content of nitrate nitrogen.

The conditions on plots 65N and 65S of the phosphate area were studied. Among the miscellaneous crops, cabbage (Enkhuizen variety), carrots, buckwheat, and corn received attention. The cabbage plants were set June 15; carrot seed was sown May 11; buckwheat was sown May 14; and corn was sown May 10. Table 4 gives the current nitrate-nitrogen and total nitrogen contents of these crops, as well as the relative yields and the quantity of nitrogen applied in fertilizer. On July 16 side dressings of 40 and 60 pounds per acre of nitrogen were made to plots 65N and 65S, respectively. The effect of this application seems to be reflected in the cabbage solutions as analyzed July 26. The low rainfall and high temperature conditions just prior to this date probably helped to produce the abnormally high value of 600 parts per million of nitrate nitrogen in plant solution.

In the case of cabbage and carrot there is not such a clear-cut correlation between solution content and fertilization, as was noted with the market-garden crops. This could hardly be expected, as the nitrogen condition on plot 65N was not considered to be suboptimum. With none of the crops can the relative yields be considered as more than directional, and inconsistencies might be expected in the solution content. This is further proven by the lack of correlation between the total nitrogen in tissue at harvest and the relative yields. It would seem that under existing conditions of soil and climate, and with such a small difference in nitrogen application, little increase in yield or reflection in solution could be expected, since the nitrogen in both cases is present in sufficient amounts.

TABLE 3.—Comparison of nitrate nitrogen in plant solution, and the quantity of nitrogen added in chemical fertilizer, market-garden experiment

Crop	Plot	Tissue examined	Relative yield	N added in chemical fertilizer (pounds per acre)	Nitrate nitrogen in plant solution on certain dates (quarts per million)										Total N in tissue on certain dates (per cent in dry matter)
Cabbage (spring)	18M(G)	Lower leaves with midribs discarded	100	100	May 28	June 17	June 25	July 9	July 21	July 30	July 19				July 19
	110M(G)		81	20	533	286	382	520	392	215	2.98				2.92
	Do.				233	130		272							July 9
Lettuce (spring)	115M(G)	Leafy	100	100	June 22	July 8					4.83				July 9
	116M(G)		79	60	417	451					3.38				Oct. 26
	Do.			60	68						2.95				Oct. 26
Beets (fall)	118M(G)	Petioles and leaves	100	90	Sept. 13	Sept. 28	Oct. 1	Oct. 11	Oct. 18		2.81				Oct. 23
	119M(G)		72	45	518	78	42	30	30		2.81				Oct. 23
	Do.				108						2.87				Oct. 23
Celery (fall)	115M(G)	Do.	100	110	Sept. 8	Sept. 30	Oct. 1	Oct. 5	Oct. 13	Oct. 14	3.20				Oct. 23
	116M(G)		87	53	517	340		308	500		2.87				Oct. 23
	Do.				326	171				205					Oct. 23

a 16 tons stable manure applied.

TABLE 4.—Comparison of nitrate nitrogen in plant solution, and the quantity of nitrogen added in otherwise optimum chemical fertilizer, phosphate experiment

Crop	Plot	Tissue examined	Relative yield	N added in chemical fertilizer (pounds per acre)	Nitrate nitrogen in plant solution on certain dates (quarts per million)										Total N in tissue on certain dates (per cent in dry matter)
Cabbage (late) <sup>a</sup>	65N	Lower leaves with midribs discarded	83	95	July 13	July 26	Aug. 19	Aug. 30	Aug. 24						
	65S		100	140	209	600	340	248	3.44						
	Do.				200	600	358	365	3.41						
Carrots	65N	Roots	100	95	July 27	Aug. 5	Aug. 18	Sept. 8	Sept. 16						
	65S		87	140	100	137	69	76	76						
	Do.				78	318	91	109	31						
Buckwheat	65N	Stems and leaves	95	95	June 16	June 28									
	65S		100	140	553	625									
	Do.				620	750									
Corn	65N	One foot of stem above upper node	98	95	July 16	July 28	Aug. 9								
	Do.		100	140	319	288									
	Do.				405	394	102								

<sup>a</sup> Enkhuizen variety.

TABLE 5.—Comparison of potassium content of plant solution, and potassium oxide added in the chemical fertilizer, potash experiment

Crop	Plot	Tissue examined	Relative yield	K <sub>2</sub> O added in chemical fertilizer (pounds per acre)	Potassium content in plant solution on certain dates (parts per million)						Total K <sub>2</sub> O in tissue on certain dates (per cent in dry matter)
					July 9	July 14	July 22	Aug. 9	Aug. 24	Aug. 10	
Turnips	110	Leaves and petioles	13	0	1,047	919	1,301	1,115	1,513	1,011	Nov. 5
	117	do	71	50	2,807	1,936	2,398	2,066	1,686	1,521	1.52
	119	do	100	150	5,140	2,869	4,253	2,450	2,626	2,311	3.21
	119	do	100	150	1,044	2,002	2,556	3,531	3,631	3,531	3.41
Cabbage	116	Lower leaves with midribs discarded	17	0	1,925	2,773	2,227	2,066	1,686	1,122	Sept. 8
	117	do	60	50	2,326	2,773	2,227	2,066	1,686	1,721	1.62
	119	do	100	150	4,554	4,225	3,210	2,450	2,626	3,821	3.26
	119	do	100	150	3,490	3,250	3,089	3,531	3,631	3,901	Nov. 1
Parsnips	116	Roots	20	0	2,265	1,461	1,550	1,420	1,420	1,401	Nov. 1
	117	do	71	50	3,250	2,064	2,616	2,450	2,450	1,411	1.41
	119	do	100	150	3,490	3,250	3,089	3,531	3,631	3,901	Nov. 1
	119	do	100	150	6,219	4,763	4,070	4,040	4,040	3,611	Nov. 2
Potatoes	116	Stem and tips	21	0	2,819	2,544	2,987	3,181	3,181	1,211	Oct. 11
	117	do	53	50	4,252	3,145	2,910	3,181	3,181	1,211	0.38
	119	do	100	150	6,219	4,763	4,070	4,040	4,040	3,611	1.49
	119	do	100	150	5,100	5,311	3,924	3,717	3,699	1,951	1.95
Tomatoes	116	Stem and tips	35	0	2,728	2,706	2,054	1,807	1,620	0.38	Oct. 11
	117	do	45	50	3,120	4,020	3,083	2,363	1,834	1.49	1.49
	119	do	100	150	5,100	5,311	3,924	3,717	3,699	1,951	1.95
	119	do	100	150	5,100	5,311	3,924	3,717	3,699	1,951	1.95

### CURRENT POTASSIUM CONTENT OF PLANTS STUDIED

Crops were planted on the potash experiment on the following dates: Turnips, seed, May 13; cabbage, plants, June 14; parsnips, seed, April 30; potatoes, tubers, May 3; tomatoes, plants, May 19.

In Table 5 the current potassium content of the plant solutions, the total  $K_2O$  in the tissue, the relative yields, and  $K_2O$  fertilization are given. It will be seen that the current amounts of potassium and the total  $K_2O$  in tissue quite consistently follow the fertilization, and that the relative yields correlate. As has been shown in an earlier paper,<sup>7</sup> the highest amounts of potassium in the plant solutions appear during the high temperature period, July 15 to July 29. This seems to explain the highest values of potassium which occurred with these crops.

### CURRENT PHOSPHATE-PHOSPHORUS CONTENT OF PLANTS STUDIED

The planting dates of the crops analyzed on the phosphate experiment were: Turnips, seed, May 13; carrots, seed, May 11; corn, seed, May 10; cabbage (Ballhead), plants, June 22.

In Table 6 the current phosphate phosphorus and the total  $P_2O_5$  found in the tissue during the season and at harvest, are shown, as well as the relative yields and the  $P_2O_5$  fertilization.

It has been the experience of this station that with the same crops grown under these conditions, marked differences in yield, except with carrots, resulted between plots 55N and 65N. The general appearance of the above-ground portions of these crops confirmed such observations in 1926. This is reflected in the relative yields of corn and cabbage, but with turnips the relationship is not clearly shown. The current phosphate phosphorus seems to have followed the  $P_2O_5$  applied, and the percentage of total  $P_2O_5$  in the dry tissue was also consistent.

### PLANT SOLUTION AS RELATED TO FERTILIZER APPLIED

From the foregoing it may be seen that the current amounts of nitrate nitrogen, potassium, and phosphate phosphorus in the plant solutions are influenced materially by the amounts of N,  $K_2O$ , and  $P_2O_5$  supplied the crops in chemical fertilizers, and that, in general, these two amounts directly correlate. Some such relationship, although hardly as consistent, appears with total N,  $K_2O$ , and  $P_2O_5$  in the dry tissue sampled both during growth and at harvest.

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<sup>7</sup> GILBERT, B. E., McLEAN, F. T., and ADAMS, W. L. Op. cit.

TABLE 6.—Comparison of phosphate-phosphorus content of plant solution, and  $P_2O_5$  added in chemical fertilizer, phosphate experiment

Crop	Plot	Tissue examined	Relative yield	$P_2O_5$ added in chemical fertilizer (pounds per acre)	Phosphate-phosphorus content in plant solution on certain dates (parts per million)						Total $P_2O_5$ in tissue on certain dates (per cent in dry matter)	
					June 26	July 20	Aug. 3	Aug. 16	Sept. 1	Sept. 14	Aug. 4	Oct. 18
Turnips	55N	Roots	95	50	0.0	9.0	2.0	2.03	5.4	9.6	0.50	0.16
	65N	do.	100	150	12.5	50.0	31.0	13.8	20.8	11.7	1.24	0.76
Carrots	55N	do.	97	80	25.0	13.4	Aug. 18	Sept. 3	Sept. 16		Sept. 20	
	65N	do.	100	180	31.0	15.5	18.0	17.0	11.7		0.06	
Corn	55N	One foot of stem above upper node	81	50	July 16	July 28						
	65N	do.	98	150	33.3	26.8						
Cabbage (late) <sup>a</sup>	55N	Lower leaves with midribs discarded	57	50	Aug. 20	Aug. 31	Sept. 13				Aug. 24	Oct. 4
	65N	do.	100	150	.86	4.45	1.38				0.45	0.43
					1.11	5.20	1.56				0.79	0.73

<sup>a</sup> Ballhead variety.

## DEGREE OF FLUCTUATION OF MINERAL NUTRIENTS IN PLANT SOLUTIONS

It is generally accepted that the relative metabolic needs of plants for the three mineral nutrient elements herein discussed are different. Hence it is to be expected that the plant solution would reflect this difference. This is the case. A comparison of the values given for each ingredient in Tables 3 to 6, shows that the standard deviations<sup>8</sup> of the ratios of the highest to the lowest values from the mean, are: Potassium, 0.84; phosphate phosphorus, 2.63; nitrate nitrogen, 4.09. Thus, the fluctuation of potassium during the growing season was smallest and of nitrate nitrogen greatest. This fluctuation must be considered as the resultant of the supply and demand of each ingredient; and as supply is regulated by soil, fertilizer applications, weather, and specific permeability of root membranes, it doubtless plays as great a part as do metabolic demands in determining the nutrient level in the plant solution.

## THE PLANT SOLUTION AS A MEANS OF FERTILIZER CONTROL

It is suggested that the quantities of fertilizer nutrients as found at any one time may give some indication as to the needs of the plant for these particular nutrients. The individual nutrient contents in the plant solution have been shown to correlate with fertilizer applications, and the fluctuations of the nutrient elements have been considered to some extent. It may not be possible to choose arbitrary values, which may indicate critical nutrient situations, but from the data presented in this paper the following critical concentrations are tentatively suggested: Potassium, roots or tops, 3,000 parts per million; phosphate phosphorus, roots, 20 parts per million; nitrate nitrogen, blades of leaves, 300 parts per million.

It is recognized that these quantities can be only tentative at present, as it is not possible without further data to choose critical concentrations for individual crops, tissues, or soil situations. Further work is contemplated in this connection.

## SUMMARY

In general the current concentrations of mineral nutrients in the solutions of crop plants were found to correlate directly with applications of chemical fertilizers.

Potassium was found to show the least fluctuation during the season, nitrate nitrogen fluctuated the most, while the fluctuation of phosphate phosphorus was intermediate.

The current mineral nutrient content of the plant solution is suggested as an index of fertilizer needs, and tentative critical concentrations have been chosen for each nutrient.

<sup>8</sup> In this calculation the highest value per ingredient, plot, and crop was divided by the lowest value, thus giving a ratio. These ratios were subjected to the standard deviation formula, i. e.  $\sigma = \sqrt{\frac{\sum (f \cdot d^2)}{n}}$ .

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## INHERITANCE OF SMOOTH SEEDS IN COTTON<sup>1</sup>

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### INTRODUCTION

The presence or absence of fuzz or short hairs on the seeds of the very long staple commercial cottons is of practical interest because the separation of the lint from the seeds by the roller gin used in ginning these cottons is more difficult when the seeds are fuzzy than when they are smooth. Sea-island cotton, for which the roller gin was first used, has seeds almost devoid of fuzz, while the longest linted varieties of the Egyptian type (Sakellaridis in Egypt and Pima in Arizona) have relatively fuzzy seeds. Experience has shown that the speed of ginning is greater and the appearance of the cotton as it comes off the rolls is better in the case of a smooth-seeded than in the case of a fuzzy-seeded cotton. The development of smooth-seeded varieties of the very long staple cottons is therefore an objective of no small importance. As a guide to practical breeding work, an understanding of the inheritance of the characters dealt with is of course desirable. The purpose of this paper is to present such information as is available concerning the inheritance of smooth seededness in cotton.

Inheritance of the seed-coat character in a hybrid between an upland cotton (Holdon variety) and an Egyptian cotton (Pima variety) has been studied by one of the writers (8, p. 11, 26, fig. 41),<sup>2</sup> who also presented (8, p. 32-33) a brief summary of the results obtained by Fletcher (6), Fyson (7), Balls (2, 3), and McLendon (11). The only subsequently published contributions to our knowledge of the subject of which the writers are aware are those of Thadani (12, 13). He found (13) that in six crosses of a naked-seeded form of upland cotton with fuzzy-seeded upland varieties the fuzzless condition was completely dominant in  $F_1$  and the  $F_2$  segregation was monohybrid. In crosses between upland varieties distinguished by a greater or less degree of fuzziness, the less fuzzy condition appeared to be dominant, this being contrary to the conclusion of Balls (2) and McLendon (11). On the other hand, crosses of completely fuzzy upland cottons with partly fuzzy Egyptian cottons showed dominance of the more fuzzy (upland) condition in  $F_1$ , with  $F_2$  distributions indicating that several factors are involved.<sup>3</sup> An exception was noted in a cross of a completely fuzzy-seeded upland with the nearly smooth-seeded Yuma variety of Egyptian cotton, the nearly smooth-seeded

<sup>1</sup> Received for publication Mar. 30, 1927; issued September, 1927. The investigation which is the principal subject of this paper was conducted at the United States Field Station, Sacaton, Ariz. The results obtained from crosses made at Greenville, Tex., by W. W. Ballard, of the Office of Cotton, Rubber, and Other Tropical Plants, also are considered on the basis of data furnished by Mr. Ballard. The photographs from which the illustrations were prepared were made by Robert L. Taylor of the same office.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 217.

<sup>3</sup> The results were therefore like those obtained by Kearney (8) with a similar cross.

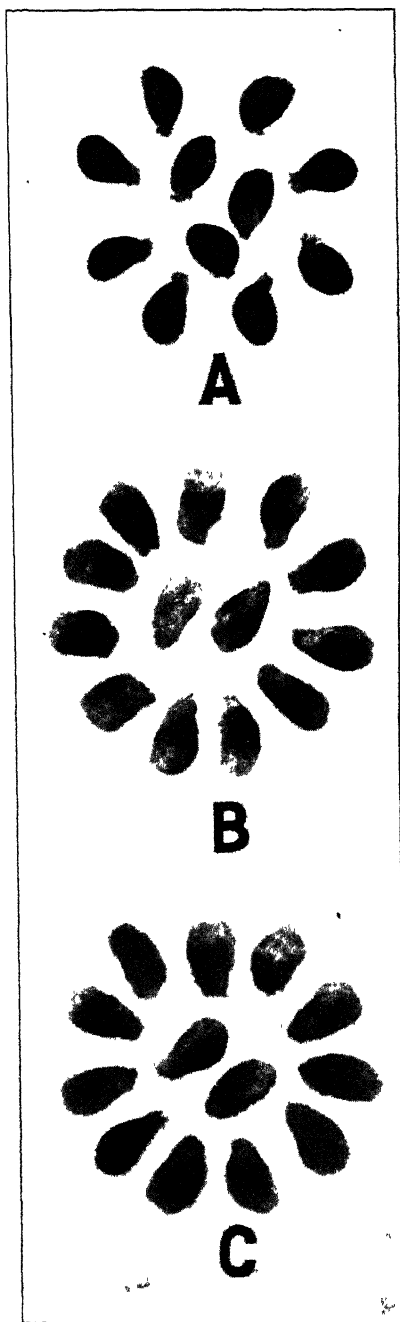


FIG. 1.—Seeds of the Pima parental families: A, the smooth-seeded family (P-SS-7) with fuzz greatly reduced and sharply localized; B, a moderately fuzzy-seeded family (P Parker); C, a very fuzzy-seeded family (P spotless)

condition having been dominant in  $F_1$ . The  $F_2$  segregates of this cross ranged from “absolutely naked” to “entirely fuzzy.”

#### INHERITANCE OF SMOOTH SEED-EDNESS IN THE PIMA VARIETY

Excellent material for a study of the inheritance of presence and absence of fuzz on the seeds in the Pima variety of Egyptian cotton is afforded by a smooth-seeded family (Pima 20-SS-7) which uniformly has the seeds naked except for a small tuft of short fuzz at base and apex and occasionally a trace along the raphe, and a “spotless” family which has the seeds almost completely covered with fuzz, representing about the extreme of fuzziness in the Pima variety. The average quantity and distribution of the fuzz in the two parental families is illustrated in Figure 1, A and C.

In 1923 two plants (designated A-12 and A-13) in the homozygous smooth-seeded Pima progeny 7-2-10, and two plants (designated B-8 and B-12) in the homozygous smooth-seeded Pima progeny 7-2-11, were chosen as female parents. Flowers on these plants were emasculated and pollinated with pollen from one plant in each of the two homozygous “spotless” and fuzzy-seeded Pima progenies, 20-19 and 21-22.<sup>4</sup> The male parent in progeny 20-19 was designated H and the male parent in progeny 21-22 was designated J. The combinations were made as follows:

$$\begin{array}{ll} A\ 12 \times H & A\ 13 \times J \\ B\ 8 \times H & B\ 12 \times J \end{array}$$

Each combination was represented by an  $F_1$  progeny grown in 1924, and flowers were selfed on several plants in each of these progenies.

<sup>4</sup> These were  $F_2$  progenies, recessive for petal spot and uniformly fuzzy seeded, which were derived from crosses of “spotless” with normally spotted Pima families, as described by one of the writers in another paper (9).

From the resulting seed 17  $F_2$  progenies were grown in 1925. These were:

A $\times$ H- 4	A $\times$ J- 3	B $\times$ H-1	B $\times$ J-12
A $\times$ H- 6	A $\times$ J- 4	B $\times$ H-3	B $\times$ J-13
A $\times$ H- 7	A $\times$ J- 9	B $\times$ H-4	B $\times$ J-14
A $\times$ H-21	A $\times$ J-18	B $\times$ H-5	B $\times$ J-15
		B $\times$ H-7	

Flowers were selfed on numerous plants in the  $F_2$  progenies, and from the resulting seed 30  $F_3$  progenies were grown in 1926. These were:

A $\times$ H 4- 3*	A $\times$ H 21-22*	B $\times$ H 5- 1
A $\times$ H 4- 7*	A $\times$ H 21-26	B $\times$ H 5- 7*
A $\times$ H 4-24	A $\times$ J 4-15	B $\times$ J 12-11
A $\times$ H 6- 4*	A $\times$ J 9- 1*	B $\times$ J 13-19
A $\times$ H 6-19	A $\times$ J 9-12	B $\times$ J 13-23*
A $\times$ H 7-14*	A $\times$ J 18- 5	B $\times$ J 14- 2
A $\times$ H 7-15*	A $\times$ J 18-23	B $\times$ J 14-21
A $\times$ H 7-19	B $\times$ H 1-14	B $\times$ J 14-23
A $\times$ H 7-21	B $\times$ H 3-10*	B $\times$ J 15-11
A $\times$ H 21- 1	B $\times$ H 3-11	B $\times$ J 15-27

Ten of these progenies (indicated by asterisks) had a fuzzy-seeded  $F_2$  parent, while the other 20 progenies had a smooth-seeded parent.

#### DOMINANCE OF SMOOTH SEEDEDNESS IN $F_1$

The whole population numbered 92, the four  $F_1$  progenies containing from 21 to 24 plants each. All of these plants were similar to the smooth-seeded parent in the quantity and distribution of the fuzz on the seeds. In other words, the approximately naked condition of the seed coat of the smooth-seeded strain was completely dominant to the very fuzzy condition characteristic of the other parent of the cross.

#### SEGREGATION IN $F_2$

The individual plants of the  $F_2$  progenies were readily classified as "smooth" or "fuzzy," since in nearly all cases their seeds were either as nearly naked as in the smooth-seeded parental family (fig. 1, A) or as fuzzy as in the fuzzy-seeded parent (fig. 1, C). The very few plants whose seeds showed appreciably more fuzz than has been observed in the former family, although less than is characteristic of the latter family, were classified as "fuzzy." The number of such individuals was far too small to affect the result.

The segregation in  $F_2$  is shown in Table 1. Since the numbers of plants in the several progenies were small, all progenies of each combination (A  $\times$  H, etc.) are taken as one array in presenting the results.<sup>5</sup> It is obvious that the segregation is monohybrid, the fuzzy-seeded condition behaving as a simple recessive. The departure of the percentage of fuzzy-seeded individuals from the expected 25 per cent in no case amounted to more than 2.6 times the probable error of the departure, and for the whole  $F_2$  population of 381 plants taken as one array the departure was only 1.8 times its probable error.

<sup>5</sup> For the 17  $F_2$  progenies,  $\chi^2$  of the departure of the observed distribution from the distribution expected on the basis of a 3:1 ratio ranged from 0 to 5.4 the latter value indicating a chance of 1 in 50 that the departure was fortuitous. Only four of these progenies gave a  $\chi^2$  greater than 0.7.

TABLE 1.—Segregation in respect to the seed-coat character in  $F_2$  of crosses between a smooth-seeded and a fuzzy-seeded family of Pima cotton

Combination	Number of progenies	Number of plants			Percentage of fuzzy-seeded plants
		Total	Smooth seeded	Fuzzy seeded	
A $\times$ H.....	4	102	74	28	27.4 $\pm$ 2.98
A $\times$ J.....	4	99	66	33	33.3 $\pm$ 3.19
B $\times$ H.....	5	72	56	16	22.2 $\pm$ 3.34
B $\times$ J.....	4	108	80	28	25.9 $\pm$ 2.84
Total.....	17	381	276	105	27.5 $\pm$ 1.54

INHERITANCE OF CHARACTER IN  $F_3$ 

Progenies of 20 smooth-seeded and of 10 fuzzy-seeded  $F_2$  individuals were grown in 1926. The 10 progenies of recessive  $F_2$ 's contained from 8 to 15 plants each, the combined  $F_3$  population from fuzzy-seeded  $F_2$  plants numbering 110 plants, all of which had seeds similar in degree of fuzziness to the fuzzy-seeded parental race. The recessive nature of the fuzzy condition is therefore completely established.

Of the 20  $F_3$  progenies of smooth-seeded  $F_2$  plants, only two (A  $\times$  J 18-23 and B  $\times$  H 5-1) contained no fuzzy-seeded individuals, and the numbers were too small (17 and 19, respectively) to make it certain that they were homozygous for smooth seediness, especially since two other progenies of smooth-seeded  $F_2$ 's, containing 19 and 20 plants, respectively, each included only one fuzzy-seeded individual. Assuming, however, that the progenies in which there were no fuzzy-seeded plants were really homozygous, it is to be noted that there were only two instead of the expected  $20 \div 3 = 6.67$  such progenies. From the value of  $\chi^2$  (4.9) it appears that the chances are 1 in 37 that the departure from the expected number of homozygous progenies is fortuitous.

TABLE 2.—Segregation in respect to the seed-coat character in 18 heterozygous  $F_3$  progenies of crosses between a smooth-seeded and a fuzzy-seeded family of Pima cotton

Combination	Progeny	Number of plants			Percentage of fuzzy-seeded plants	$\chi^2$	P
		Total	Smooth seeded	Fuzzy seeded			
A $\times$ H.....	4-24	19	12	7	36.8	1.421	0.233
	6-19	18	16	2	11.1	1.852	.174
	7-19	20	14	6	30.0	.267	.605
	7-21	18	13	5	27.8	.074	.786
	21-1	19	18	1	5.3	3.948	.047
	21-26	22	17	5	22.7	.060	.806
A $\times$ J.....	4-15	16	9	7	43.7	3.000	.083
	9-12	17	10	7	41.1	2.372	.124
	18-5	16	13	3	18.7	.333	.564
B $\times$ H.....	1-14	19	10	9	47.3	5.070	.025
	3-11	21	17	4	19.0	.396	.529
B $\times$ J.....	12-11	20	14	6	30.0	.267	.605
	13-19	16	11	5	31.2	.333	.564
	14-2	20	19	1	5.0	4.267	.039
	14-21	19	13	6	31.5	.439	.508
	14-23	19	14	5	26.3	.017	.897
	15-11	19	17	2	10.5	2.122	.145
	15-27	19	17	2	10.5	2.122	.145

The segregation in the 18 heterozygous  $F_3$  progenies is shown in Table 2. Since the progenies were small and in some of them the percentage of fuzzy-seeded individuals was very low, it seems best to judge the goodness of the fit by the probability, as indicated by  $\chi^2$ , of the departure of the observed distribution from that expected with a 3:1 ratio. The chance of a fortuitous departure from the expectation as great as that shown by the progeny which gave the highest value for  $\chi^2$  (5.07) is 1 in 40.

In Table 3 the several heterozygous  $F_3$  progenies of each combination are taken as one array and the percentages of fuzzy-seeded plants are given for these larger populations. In none of them is the departure from the expected 25 per cent equal to twice its probable error, and for the entire heterozygous  $F_3$  population the departure is only one-fourth its probable error.

TABLE 3.—Segregation in respect to the seed-coat character in groups of heterozygous  $F_3$  progenies representing each of the crosses between a smooth-seeded and a fuzzy-seeded family of Pima cotton

Combination	Number of progenies	Number of plants			Percentage of fuzzy-seeded plants
		Total	Smooth seeded	Fuzzy seeded	
A $\times$ H.....	5	94	73	21	22.3 $\pm$ 2.91
A $\times$ J.....	4	71	49	22	31.0 $\pm$ 3.74
B $\times$ H.....	2	40	27	13	32.5 $\pm$ 5.09
B $\times$ J.....	7	132	105	27	20.4 $\pm$ 2.37
Total.....	18	337	254	83	24.6 $\pm$ 1.58

#### INHERITANCE OF SMOOTH SEEDEDNESS IN CROSSES AMONG VARIOUS FAMILIES

Further indications of the manner of inheritance of the smooth and fuzzy condition of the seed coat were obtained by nine combinations made in 1924 among various families, some of which represented different species (Egyptian and upland cottons). With one exception (combination 3), each combination was represented by two crosses, different individuals of the respective parental families having been used as parents of each cross, except that in combination 8 the same Ballard individual served as the male parent of both crosses.  $F_1$  progenies representing both crosses of each combination were grown in 1925. From selfed seed of a single individual in one of the  $F_1$  progenies, an  $F_2$  progeny of each combination was grown in 1926.

Five open bolls were collected from each plant in the parental and  $F_2$  progenies of 1926, and the seed cotton was ginned with care to remove the lint as fully as possible without disturbing the fuzz. The seeds, freed from adhering lint, neps, and trash, were placed in transparent glass vials, one for each plant, and with the samples in this convenient form classification of the populations was readily accomplished.

#### CHARACTERISTICS OF THE PARENTAL FAMILIES

Of the parental families involved in these crosses, the first three herein described were of the Pima variety of Egyptian cotton (*Gossypium barbadense*  $\times$  ?) and the other four were upland cottons (*G. hirsutum*).

## PIMA SMOOTH SEED (P-SS-7)

The parental progeny was of the third inbred generation from a plant of the Pima variety selected at Sacaton in 1920 by Walter F. Gilpin and characterized by the almost complete absence of fuzz on the seeds. It proved to be homozygous for this character, no other kind of seeds having been found on hundreds of its descendants examined during the past six years. The particular individual which gave rise to the progeny grown in 1924 belonged to the progeny of 1923 (7-2-10) which furnished the "A" parents of the combinations described in the earlier pages of this paper. Different individuals in progeny 7-2-10-17 of 1924 were used as one of the parents of combinations 1, 2, 7, and 9, respectively. (Table 4.) Selfed seed of plant 5, the female parent of combination 7, was planted in 1926 and gave a progeny of eight plants. All of these were alike and showed the condition typical of this family, the fuzz being confined to a pronounced but sharply limited tuft at the small end of the seed and often a smaller tuft at the large end, with an occasional trace along the raphe. (Fig. 1, A.)

## PIMA PARKER

The parental progeny was of the third inbred generation from an individual of the Pima variety selected by Warren Parker at Phoenix, Ariz. Different individuals in the progeny of 1924 (P. Parker 6-10-17) were used as one of the parents of combinations 1, 2, 6, and 8, respectively. (Table 4.) Selfed seed of plant 15, the female parent of combination 1, was planted in 1926 and gave a progeny of 10 plants, all of which had the seeds partly covered with typical Pima fuzz, greenish or brownish in color. (Fig. 1, B.) The average degree of fuzziness in this progeny was near the average for the Pima variety, the classification having been: Grade 6, 4 plants; grade 7, 4 plants; grade 8, 2 plants. As is the rule in the Pima variety, there was considerable variation on the individual plant in the degree of fuzziness.<sup>6</sup>

## PIMA SPOTLESS

The parental progeny was a recessive  $F_4$  of one of the crosses, described in another publication (9), between representatives of a Pima family having almost no petal spot and representatives of a normally spotted family of the same variety. It was grown from selfed seed of plant 34 in the  $F_3$  progeny (21-22), which furnished the "J" parents of the combinations described in earlier pages of this paper. Both the spotless and the normally spotted parents of the original cross were fuzzy seeded, and the descendants have exhibited this condition uniformly. Different individuals in  $F_4$  progeny 21-22-34 of 1924 were used as one of the parents of combinations 4 and 5, respectively. (Table 4.) An  $F_4$  progeny grown in 1925 from selfed seed of another individual in the  $F_4$  progeny comprised 18 plants, the seeds of all of which were decidedly more fuzzy (grade 8 to 9) than the average for Pima. Only two plants survived in the  $F_5$  progeny grown in 1926, but these had seeds similar to those of the  $F_3$  plants. (Fig. 1, C.)

## BALLARD'S NAKED-SEEDED UPLAND

The writers are indebted to W. W. Ballard, the originator of this family, for the following record of its performance at Greenville, Tex. The original naked-seeded selection, in a population of upland cotton grown from seed obtained from Cuba, was made at Greenville in 1916. A progeny grown in 1917 from open-pollinated seed from this plant contained, as would be expected, some fuzzy-seeded individuals. Open-pollinated seed was saved from plants in the 1917 progeny which had naked seeds and very sparse lint. A progeny, grown in 1919 from seed of the most nearly lintless of the 1917 selections, contained some fuzzy-seeded plants and showed variation in the quantity of lint. Flowers were selfed on the plants grown in 1919, and selfed seeds from the plants most nearly lintless were used in planting three progenies grown in 1920. No fuzzy-seeded plants appeared in the 1920 progenies, and in one of them most of the plants were almost lintless. Selfed seed from two plants in the latter progeny gave rise to two progenies grown in 1921. Neither of these contained any fuzzy-seeded individuals.<sup>7</sup> Seed from a plant in one of these progenies was sent to

<sup>6</sup> In the type sample of grade 7 for Pima cotton the seeds have from about one-fourth to nearly all of the surface covered with fuzz.

<sup>7</sup> No record was preserved of the size of the progenies grown in 1920 and 1921, but it is Mr. Ballard's recollection that each of them contained about 20 individuals.

Sacaton, Ariz., and two sister plants in the same progeny were used as parents of crosses with two plants of Lone Star made by Mr. Ballard and hereafter described in this paper.<sup>5</sup> No fuzzy seeds appeared in two succeeding inbred generations of this family at Greenville, although the population grown in 1923 comprised 63 individuals.

The seed from Greenville was planted at Sacaton in 1924, and seven plants survived, all of which were recorded as having fuzzless seeds. (Fig. 2.) Different individuals in this population were used as one of the parents of combinations 3, 7, and 8, respectively. (Table 4.) From selfed seed of two of the plants, one of which (No. 1) had served as the Ballard parent of combination 3, progenies of four and five individuals, respectively, were grown in 1925. These plants were uniformly almost fuzzless, showing only a minute tuft of fuzz at the small end of the seed. Selfed seed of a plant (No. 1-2) in the 1925 progeny of plant No. 1 gave rise to a progeny of 10 plants grown at Sacaton in 1926.

The behavior at Greenville, rather than the absence of fuzzy seeds in the very small populations grown at Sacaton in 1924 and 1925, led the writers to believe that this family is homozygous smooth seeded, and on this assumption it was used in crosses with other families in 1924. It was a surprise, therefore, to find the 1926 Ballard progeny segregating in the proportion of seven naked or nearly naked to three fuzzy. The several types of segregates were exactly duplicated in  $F_2$  of the cross Ballard  $\times$  Lone Star (combination 3). The fuzzy-seeded individuals were very similar to Lone Star (fig. 3, A) having seeds completely covered with typical upland fuzz. (Fig. 4, D). Of the smooth-seeded segregates, two had approximately the quantity of fuzz (chiefly at the small end) characteristic of the smooth-seeded Pima family (fig. 4, C), while the other five had the seeds entirely or almost entirely devoid of fuzz (fig. 4, A and B).

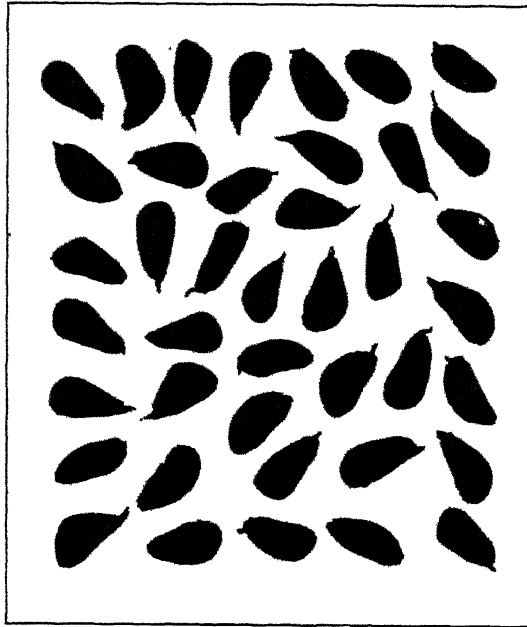


Fig. 2.—Seeds of the Ballard upland parental family, showing the typical fuzzless condition and the long slender funiculus or seed stalk.

The possibility is not excluded, in spite of the precautions taken at Sacaton to avoid such errors, that seeds resulting from natural cross-pollination of unprotected flowers on plant No. 1 of 1924 may have been gathered accidentally with the selfed seeds of this plant and that a plant grown in 1925 from such a cross-pollinated seed was the mother of the 1926 progeny. This seems a reasonable explanation of the segregation observed in progeny 1-2 of 1926, because, as will be shown in discussing combination 3, of which plant No. 1 was the Ballard parent, the result in  $F_1$  affords evidence that this plant was homozygous for smooth seeds.

Yet, although we may assume homozygosity of the particular individual of the 1924 Ballard progeny which gave rise to the segregating Ballard progeny of 1926, it will be seen from the results in  $F_2$  of another cross (combination 7) that not all of the plants in the 1924 progeny of the Ballard family were homozygous. It is probable, therefore, that the seeds sent from Greenville and planted at Sacaton in 1924 included some which had resulted from accidental cross-pollination with a fuzzy-seeded upland cotton.

<sup>5</sup> It may be noted here that the  $F_1$ 's of these crosses were uniformly smooth seeded, but the populations were too small to afford proof that the Ballard parents were homozygous for smooth seeds.

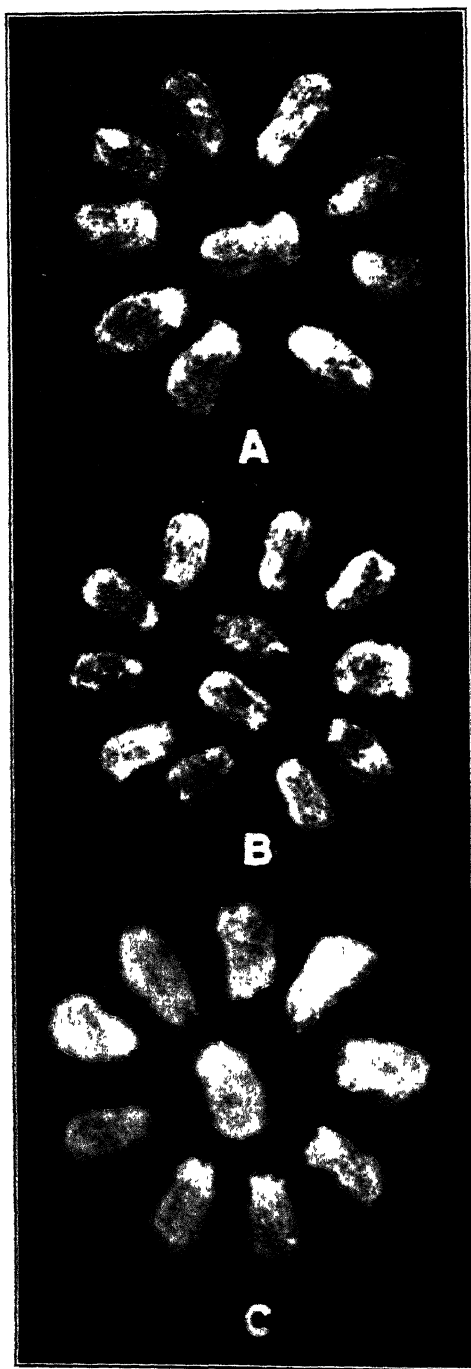


FIG. 3.—Seeds of the homozygous fuzzy-seeded upland parental families. A, Lone Star; B, Acala; C, Holdon

There is a tendency in this family, as is often the case in naked-seeded variations of upland cotton, to suppression of the long hairs or lint as well as of the shorter hairs or fuzz. (See fig. 10, p. 213.)<sup>9</sup> None of the smooth-seeded segregates in the 1926 progeny at Sacaton was entirely lintless, but some individuals had very little while others had fairly abundant lint. Another characteristic of the typical naked seeds (fig. 2) is the long and very slender stalk (funiculus). In this, as in other seed characters, the naked-seeded variants of upland cotton resemble the "Hindi" cotton which occurs as a weed in fields of cultivated cotton in Egypt (5, p. 12).

#### LONE STAR UPLAND

A population of eight individuals, from selfed seed of a plant grown at Sacaton in 1919, was grown in 1924. Different individuals in this progeny served as one of the parents of combinations 3, 6, and 9, respectively. (Table 4.) Selfed seed of another individual (No. 7) in this population gave rise to a progeny of nine individuals grown in 1925, and from selfed seed of one of the plants (7-4) in the 1925 progeny a progeny of nine individuals was grown in 1926. In all three generations all individuals showed the condition characteristic of the Lone Star variety and or most upland cottons, the seeds being covered with whitish fuzz much longer than that of Pima Egyptian. (Fig. 3, A.) The density of the fuzz varies, however, there being areas on some of the seeds of nearly all plants where the fuzz is so sparse that the dark seed coat shows through. On one plant of the 1926 progeny all of the seeds were thus characterized.

#### ACALA UPLAND

In a progeny of the third inbred generation, grown in 1924, one of

<sup>9</sup> In the segregates of combinations of which this family furnished one of the parents, it was not always easy to make a sharp distinction between lint hairs and fuzz hairs. The difference between the two kinds of hairs is stated by Balls (4, p. 83) as follows: "The hairs of the fuzz are distinguishable from those of the lint by their much greater diameter, even in the earliest stages of their development. They are as a rule about twice the diameter of a lint hair, or more. They arise in much the same way, at the same time, and from the same layer of cells."

the plants was used as the male parent of combination 4. (Table 4.) From selfed seed of the same individual (No. 22) a progeny of 11 plants was grown in 1925, and selfed seed of a plant in this progeny (22-5) gave rise to a progeny of 10 individuals grown in 1926. Both 1925 and 1926 progenies were homozygous for fuzzy seeds, a condition typical of the Acala variety. The fuzz, whitish in color, was shorter than in Lone Star and covered the seed completely, although usually less densely on the faces than on the ends of the seeds. (Fig. 3, B.)

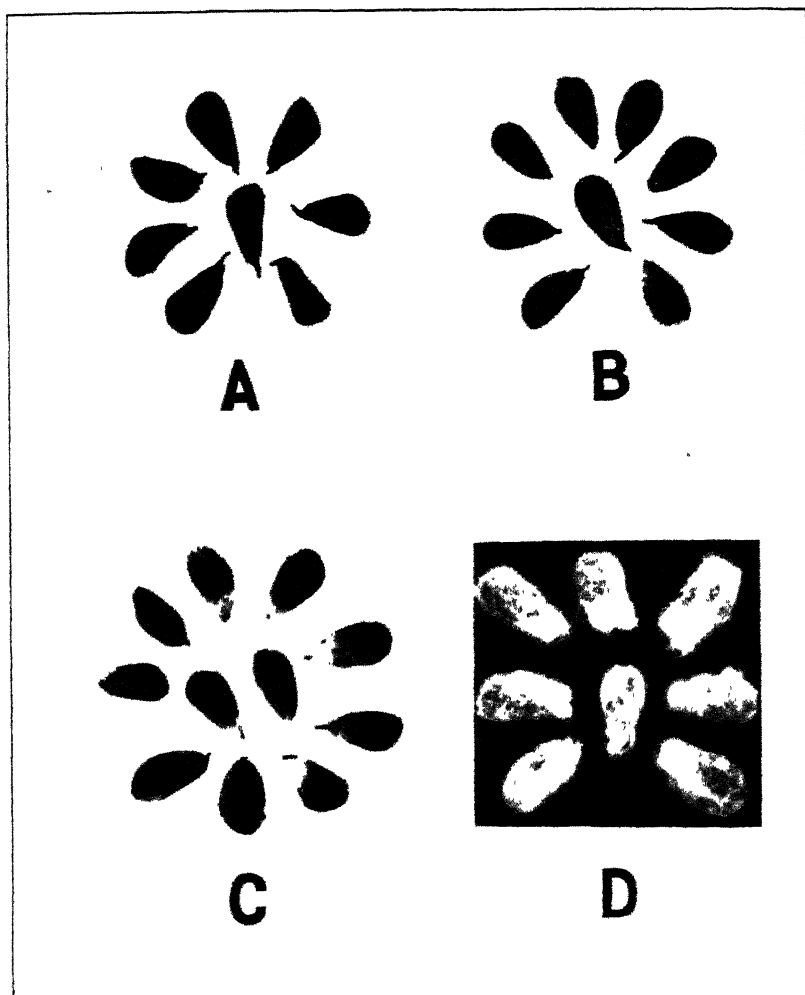


FIG. 4.—Seeds of  $F_2$  plants of combination 3, a cross between a smooth-seeded upland (Ballard) and a fuzzy-seeded upland (Lone Star), showing the several types of segregates: A, fuzzless; B, nearly fuzzless, C, with localized fuzz, classed as smooth seeded; D, completely fuzzy

#### HOLDON UPLAND

No record was made of the seed coat condition in the progeny of 1924 which furnished the male parent of combination 5 (Table 4), but it was of the fifth inbred generation, and its ancestors were uniformly fuzzy seeded. The 10 plants of a progeny grown in 1926 were typical in having the seeds completely covered with nearly pure white fuzz, much longer and denser than in the Lone Star and Acala parental families. (Fig. 3, C.)

Comparing the several parental families, it will be noted that the seeds of the fuzzy-seeded uplands (fig 3) are more completely covered

and have longer fuzz than the seeds of the fuzzy-seeded Pimas (fig. 1, B and C). On the other hand, the seeds of the Ballard upland family (fig. 2) are more nearly naked than the seeds of the smooth-seeded Pima family (fig. 1, A).

#### BEHAVIOR OF THE CHARACTER IN THE HYBRID POPULATIONS

The results in  $F_1$  and  $F_2$  are presented in Table 4. The hybrid populations were too small to afford conclusive evidence of the manner of inheritance of this seed character, but the data are useful as suggesting lines of future investigation.

TABLE 4.—*Inheritance of the seed-coat character in various combinations of Pima Egyptian and upland cottons*

Character of seed coat of individuals which served as parents of the crosses	Combination	$F_1^a$		$F_2^b$		
		Smooth	Fuzzy	Smooth	Fuzzy	(?)
Smooth $\times$ fuzzy (Pima $\times$ Pima).	(1) P-SS-7 $\times$ P Parker.....	7	0	19	7	-----
	(2) P Parker $\times$ P-SS-7.....	6	0	15	6	-----
Smooth $\times$ fuzzy (upland $\times$ upland).	(3) Ballard $\times$ Lone Star.....	8	0	8	0	-----
		12	0	22	5	-----
Fuzzy $\times$ fuzzy (Pima $\times$ upland).	(4) P spotless $\times$ Acala.....	0	7	0	25	1
	(5) P spotless $\times$ Holdon.....	0	9	0	21	-----
	(6) P Parker $\times$ Lone Star.....	0	5	0	23	-----
		0	9	0	8	-----
Smooth $\times$ smooth (Pima $\times$ upland).	(7) P-SS-7 $\times$ Ballard.....	8	0	20	3	-----
		10	0	12	9	1
Fuzzy $\times$ smooth (Pima $\times$ upland).	(8) P Parker $\times$ Ballard.....	10	0	2	11	1
		9	0			
Smooth $\times$ fuzzy (Pima $\times$ upland).	(9) P-SS-7 $\times$ Lone Star.....	0	10			
		0	10			

<sup>a</sup> Except in combination 3, two  $F_1$  progenies, representing crosses between different individuals of the same parental families, were grown in 1925.

<sup>b</sup> Each  $F_2$  progeny was grown from selfed seed of a single plant in the  $F_1$  progeny which is on the same line in the table.

Combinations 1 and 2 (Table 4) are reciprocal crosses between representatives of the smooth-seeded Pima family which furnished one of the parents of the crosses ( $A \times H$ , etc.) described in the earlier pages of this paper and a fuzzy-seeded Pima family (P Parker) not related to the "spotless" family which furnished the fuzzy-seeded parents of crosses  $A \times H$ , etc. The inheritance in combinations 1 and 2 is similar to that in the crosses previously described—dominance of smooth seededness in  $F_1$  and monohybrid segregation in  $F_2$ .

In the quantity, character, and distribution of the fuzz,  $F_1$  and the smooth-seeded segregates in  $F_2$  were like the smooth-seeded parental family, P-SS-7 (fig. 1, A), and the fuzzy-seeded segregates in  $F_2$  were like the fuzzy-seeded parental family, P Parker (fig. 1, B). The results suggest, although they do not prove, that the "Parker" family of Pima has the same major factor for seed fuzziness as the "spotless" family which furnished the fuzzy-seeded parents of combinations  $A \times H$ , etc.

Combination 3 (Table 4) is a cross between a smooth-seeded family of upland cotton (Ballard naked seeded) and a family representing the typical condition in a very fuzzy-seeded variety of upland cotton (Lone Star). From presumably selfed seed of plant No. 1, the Ballard parent of this combination, four plants were grown in

1925, and all were smooth seeded; but a progeny of 10 plants grown in 1926 from selfed seed of a plant in the 1925 progeny segregated in the proportion of seven smooth seeded to three fuzzy seeded. Yet, as was stated in the account of the Ballard parental family, this segregation may have been due to accidental planting in 1925 of a seed resulting from natural cross-pollination on plant No. 1 of 1924, in which case it does not follow that the 1924 plant itself was heterozygous. That it was, on the contrary, homozygous is indicated by the fact that in  $F_1$  of combination 3 all 12 of the plants were smooth seeded.<sup>10</sup> If the Ballard parent of this combination had been heterozygous, the Lone Star parent being homozygous recessive, half of the  $F_1$  plants should have been fuzzy seeded. The value of  $\chi^2$  (12) indicates a chance of less than one in a thousand that the absence of fuzzy-seeded individuals in  $F_1$  is fortuitous. The  $F_2$  segregation suggests a ratio of three smooth to one fuzzy, the  $\chi^2$  of 0.6 indicating a chance of 1 in 2.3 that the departure of the observed distribution from the distribution expected with this ratio is fortuitous.

Of the 22 segregates in  $F_2$ , classed as smooth seeded, 18 had seeds that were completely fuzzless or very nearly so (fig. 4, A), three had seeds with minute tufts of very short fuzz, usually at the small end of the seed (fig. 4, B), and one had seeds with somewhat more fuzz than in the smooth-seeded Pima family (fig. 4, C). The fuzzy-seeded segregates resembled the Lone Star parent in the character and distribution of the fuzz. (Fig. 4, D.)

Combinations 4, 5, and 6 (Table 4) represent interspecific hybrids involving two fuzzy-seeded Pima families and three families, typical of as many varieties, of fuzzy-seeded upland. All of these families are believed to be homozygous recessive. One of the Pima families, P spotless (fig. 1, C), is the same involved in the crosses ( $A \times H$ , etc.), described in the earlier pages of this paper, and the other, P Parker (fig. 1, B), was one of the parents of the reciprocal combinations 1 and 2. Of the three upland parental families, Acala had the least fuzzy seeds and Holdon the fuzziest seeds. (Fig. 3.)

In  $F_1$  all individuals of the three combinations resembled the upland parent in the character and distribution of the fuzz. In  $F_2$  of combination 4, 25 of the 26 plants had fuzz over the entire surface of the seed and many plants had much longer and denser fuzz than the Acala parent. The extremes of fuzziness among these 25 plants are shown in Figure 5, A and B.<sup>11</sup> In  $F_2$  of combination 5 all of the 21 plants had the seeds covered with fuzz. On several plants the fuzz was shorter and less dense than on Holdon upland, the fuzzier seeded parent, while a few plants had even longer fuzz than Holdon. The extremes of fuzziness in this progeny are shown in Figure 5, C and D. In  $F_2$  of combination 6, only 2 of the 23 plants had less fuzz than the upland parent (Lone Star), 1 plant having most of the seeds completely covered and the remaining seeds at least half covered, while the other plant resembled the Pima parent (P Parker) in the average quantity of fuzz present. Most of the remaining 21

<sup>10</sup> In addition to the  $F_1$  progeny recorded in Table 4, two other  $F_1$  progenies, representing the reciprocal combination Lone Star  $\times$  Ballard, were grown in 1925 from crosses made in 1924 between other individuals of the same parental families. These comprised, respectively, nine and five plants, all smooth seeded.

<sup>11</sup> The exceptional plant had most of the seeds as smooth as or smoother than in the smooth-seeded Pima family (fig. 1, A), whereas a few of the seeds were comparable in fuzziness to those of the Pima Parker family (fig. 1, B). The presence of this plant was almost certainly due to accident. This can not be proved, but it is highly improbable that a plant so nearly smooth seeded, alone in a progeny of 26, could have come from a cross between a fuzzy-seeded Pima and a fuzzy-seeded upland.

plants had longer and denser fuzz and several of them had much longer and denser fuzz than the fuzzier seeded (upland) parent. The range of seed fuzziness in this progeny is shown in Figure 6.

The parental phenotypes involved in combinations 4, 5, and 6 are similar to those involved in a cross between a very fuzzy-seeded upland family (Holdon variety) and a rather fuzzy seeded Egyptian family (Pima variety) described in an earlier publication (8). The seed fuzziness of the Egyptian parental family was similar to that of the

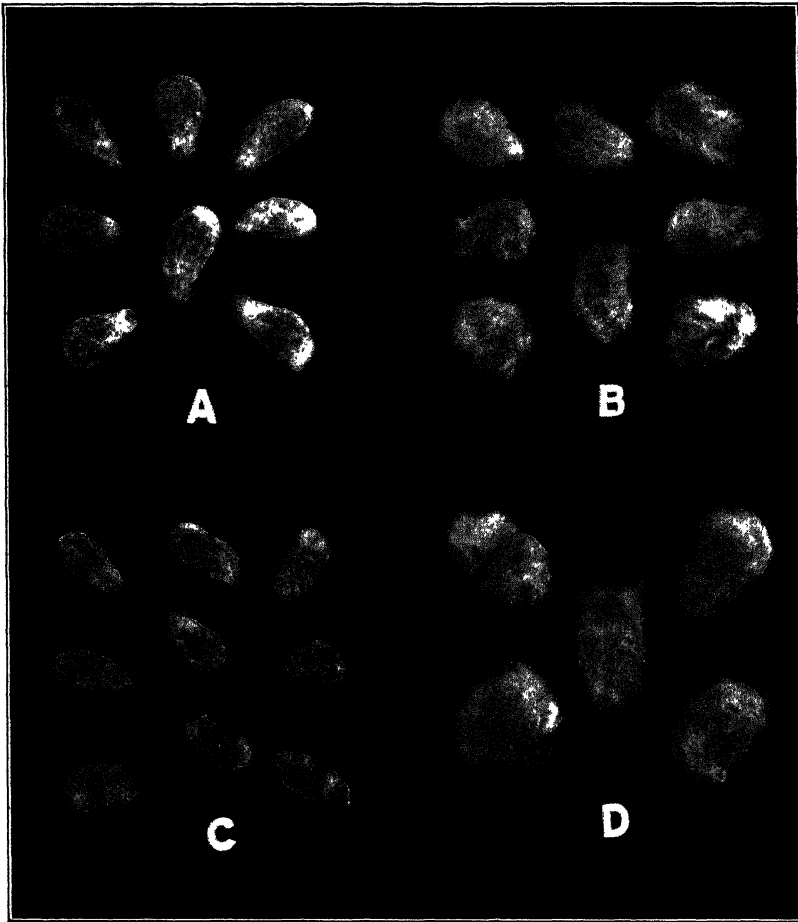


FIG. 5.—Seeds of  $F_2$  plants of crosses between a fuzzy-seeded Pima and a fuzzy-seeded upland, showing the two extremes of fuzziness: A and B, combination 4 (P spotless  $\times$  Acala); C and D, combination 5 (P spotless  $\times$  Holdon)

Pima family (P Parker) to which belonged one of the parents of combination 6, described in this paper. The upland parental family had all of the seeds covered with long white fuzz. In  $F_1$  of the cross the seeds were approximately as fuzzy as in the upland parent, but the fuzz was green or pale brown in color. The frequency distribution of  $F_2$ , as given in the publication cited (8, p. 26, fig. 41), shows that a few individuals resembled the Pima parent in degree of fuzzi-

ness. The great bulk of the population was similar to  $F_1$  and to the upland parent, and a number of plants exceeded the upland parent (fuzz much longer). Reexamination of this material confirms the conclusion that while no simple Mendelian ratio is determinable, the upland condition as to fuzziness is dominant over the Pima condition. In a total of 191  $F_2$  plants, only 7 resembled the Pima parent and none had smoother seeds. The seeds of 19 plants were

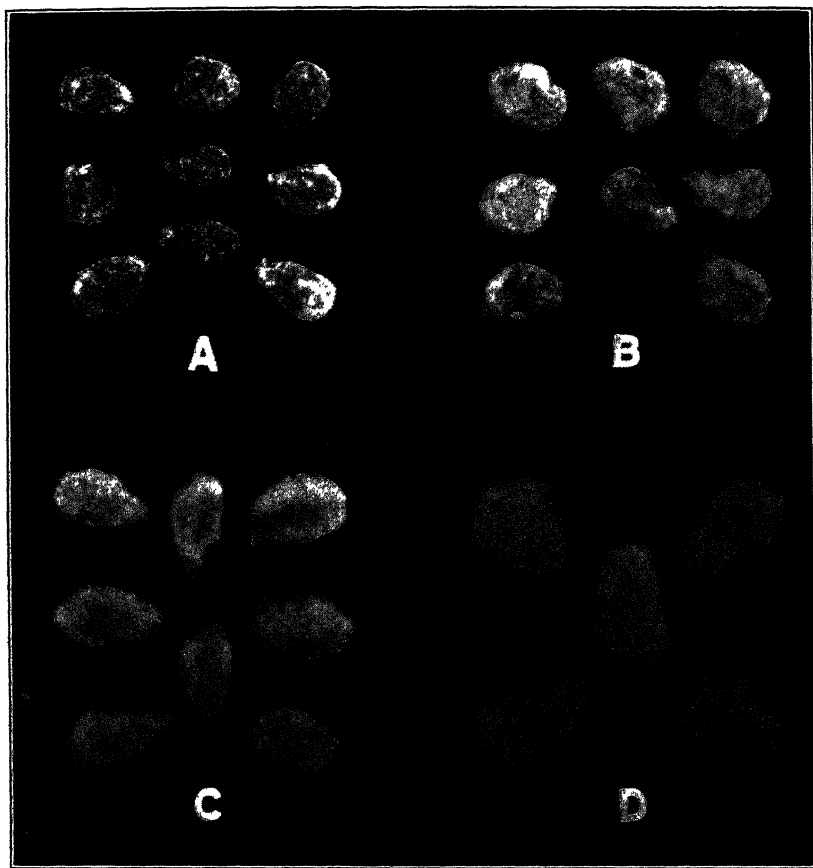


FIG. 6.—Seeds of  $F_2$  plants of combination 6, a cross between a fuzzy-seeded Pima and a fuzzy-seeded upland (P Parker  $\times$  Lone Star), showing the several types of segregates: A, the least fuzzy; B, less fuzzy than the average of the progeny; C, seed of average fuzziness; D, the most fuzzy

much fuzzier than the seeds of the Pima parent, although some of them were not completely covered. The remaining 165 plants had the seeds completely covered, and on many of these the hairs were longer than in the upland parent. There was also pronounced dominance of the greenish or brownish color of the Egyptian fuzz over the white color of the upland fuzz.<sup>12</sup>

<sup>12</sup> A large majority of the  $F_2$  individuals showed the greenish or brownish fuzz color of the Egyptian parent as compared with the whitish color of the upland parent. Considering green alone, this color was apparent in 78.4 per cent of the  $F_2$  population, while in 21.6 per cent the green color was not present, or at least was not apparent. The difficulty of classifying seeds in respect to color of the fuzz (3, p. 148) is a serious obstacle to the study of the inheritance of this color character.

Combination 7 (Table 4) represents an interspecific hybrid between a smooth-seeded Egyptian (Pima) family and the Ballard family of upland cotton. The individuals which served as the Egyptian parents belonged to the same family as the smooth-seeded

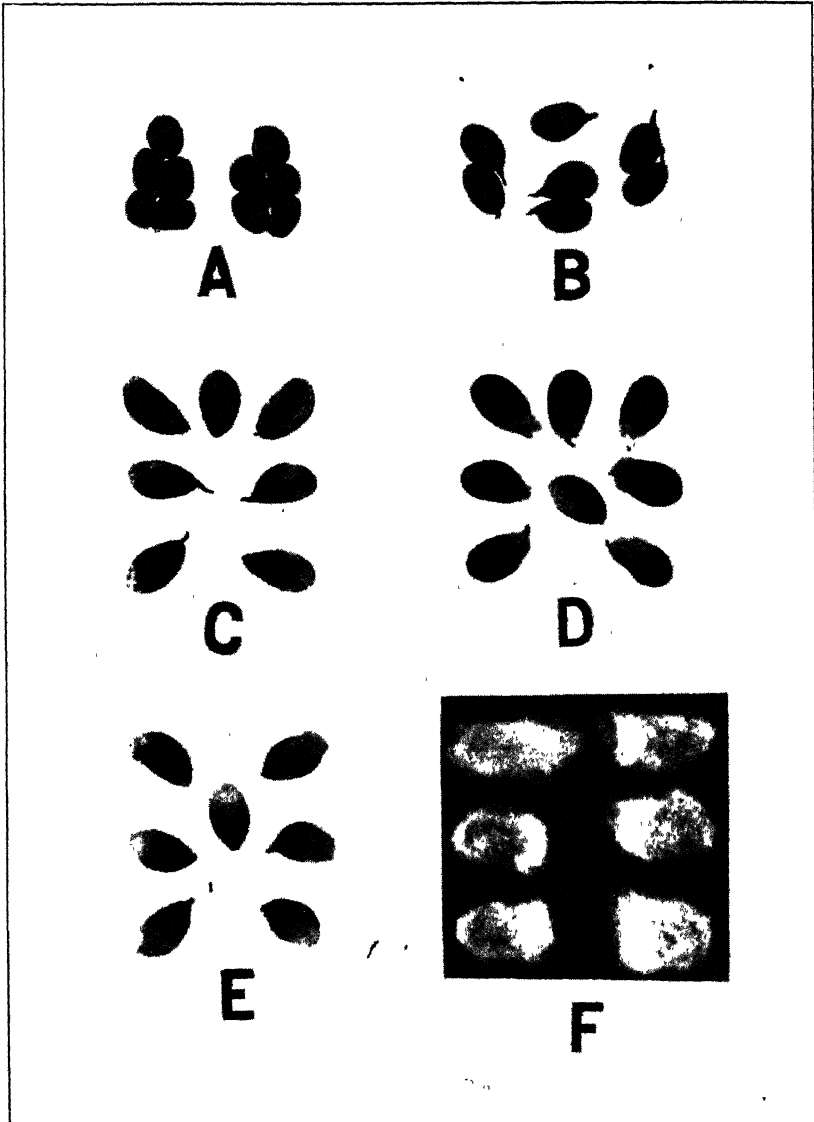


FIG. 7.—Seeds of  $F_2$  plants of combination 7, a cross between the smooth-seeded Pima family (P-SS-7) and the smooth-seeded Ballard upland family, showing the several types of segregates: A, fuzzless, seeds strongly adherent; B, fuzzless, seeds weakly adherent; C, nearly fuzzless; D, with fuzz localized at the small end of the seed; E, with fuzz localized at the large end of the seed; F, completely fuzzy. (A to E were classed as smooth seeded)

parents of the Pima  $\times$  Pima combinations A  $\times$  H, etc., and 1 and 2 (Table 4) and were unquestionably homozygous. The individuals which served as the upland parents were phenotypically smooth

seeded. Whether they were homozygous or heterozygous was not determined by growing progenies from self-pollinated seed, and the absence of fuzzy-seeded individuals in  $F_1$  does not answer the question, the Pima parents having been homozygous for smooth seeds. The segregation in  $F_2$  (20 smooth, 3 fuzzy) and the uplandlike character of the fuzzy-seeded segregates indicate, however, that the Ballard parent of the corresponding  $F_1$  progeny was heterozygous. The value for  $\chi^2$  of the departure from a 3:1 ratio in  $F_2$  is 1.75, indicating a chance of 1 in 5.4 that the departure is fortuitous.

In  $F_1$  all individuals were recorded as having the seeds completely devoid of fuzz. Of the 20 smooth-seeded segregates in  $F_2$ , 5 had the seeds entirely devoid of fuzz (fig. 7, A and B),<sup>13</sup> 12 showed the merest trace of fuzz (fig. 7, C), and 3 had a small tuft at either the large or the small end of the seed, the quantity of fuzz being about the same as in the smooth-seeded Pima family, although the hairs were longer (fig. 7, D and E). The three fuzzy-seeded segregates were similar to Lone Star in the quantity and distribution of the fuzz, but the hairs were longer than is usual in that variety. (Fig. 7, F.) It is interesting that the recessives of a hybrid between a smooth-seeded Pima and a phenotypically smooth-seeded upland showed even more than the normal upland degree of fuzziness. It is also to be noted that 17 out of 20, or 85 per cent, of the smooth-seeded  $F_2$  segregates had the naked or very nearly naked seeds of the upland (Ballard) parent.

Combination 8 (Table 4) represents the interspecific hybrid Egyptian  $\times$  upland in which the Egyptian (Pima) parents belonged to a fuzzy-seeded (homozygous recessive) family and the upland parent belonged to the Ballard family. The individual plant of the latter family used as the male parent in both crosses of this combination was phenotypically smooth seeded, but whether it was homozygous or heterozygous was not determined by planting self-pollinated seed. The fact that both  $F_1$  progenies had the seeds uniformly devoid of fuzz suggests that the upland parent was homozygous, because if it had been heterozygous a 1:1 ratio should have appeared in  $F_1$ . The values for  $\chi^2$  of the departures from this ratio in the two  $F_1$  progenies are 10 and 9, respectively, indicating chances of only about 1 in 640 and 1 in 370 that the departures are fortuitous. For the two  $F_1$  progenies, as one array,  $\chi^2 = 19$ .

The observed segregation in  $F_2$  of combination 8 (12 smooth, 9 fuzzy) throws no light upon the constitution of the upland grandparent, since the Egyptian grandparent having been recessive, the  $F_2$  progeny of a phenotypically smooth-seeded  $F_1$  plant should give a 3:1 ratio, whether the smooth-seeded (upland) grandparent was homozygous or heterozygous. The departure of the observed distribution in  $F_2$  (omitting the one individual of doubtful classification) from the distribution expected with a 3:1 ratio gives a  $\chi^2$  of 3.57, indicating a chance of about 1 in 16 that the departure is fortuitous.

Of the 12 smooth-seeded  $F_2$  segregates, 6 had the seeds devoid of fuzz or very nearly so (fig. 8, A), 5 showed minute tufts of fuzz

<sup>13</sup> Four of these five plants had some of the seeds adherent (fig. 7, A and B), although less tightly so than in typical "Kidney cotton" as described by Lewton (10). This character appeared in none of the other combinations described in this paper and was not observed in either of the families to which the parents of combination 7 belong. It is perhaps to be regarded as one of the remote ancestral characters which reappear in hybrids between different species of *Gossypium* (8, p. 39-41). The occurrence of "kidney" seed in a naked-seeded variant of upland cotton was noted by H. A. Allard (1).

(fig. 8, B), and 1 resembled the smooth-seeded Pima family in the quantity and distribution of the fuzz (fig. 8, C). The doubtful indi-

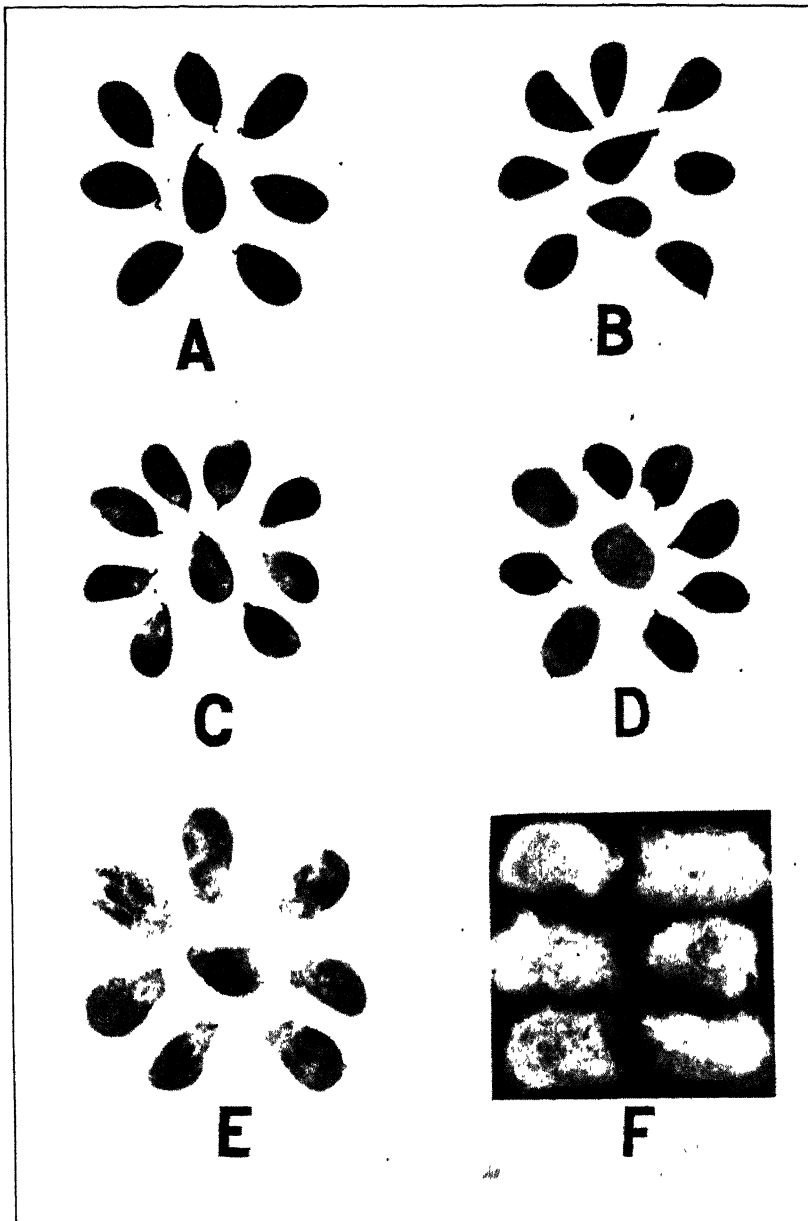


FIG. 8.—Seeds of  $F_2$  plants of combination 8, a cross between a fuzzy-seeded Pima family (P Parker) and the smooth-seeded Ballard upland family, showing the several types of segregates: A, fuzzless; B, nearly fuzzless (most of the hairs showing are adherent lint hairs); C, having localized fuzz, but classed as smooth seeded; D, uncertain, having both fuzzy and nearly fuzzless seeds; E, least fuzzy of the fuzzy-seeded class; F, most fuzzy of the fuzzy-seeded class

vidual had some of the seeds devoid of fuzz and others more or less fuzzy. (Fig. 8, D.) Of the nine fuzzy-seeded segregates, eight had

all the seeds completely covered with uplandlike fuzz (fig. 8, F), but there was considerable variation from plant to plant in the length of the fuzz and in its color, which ranged from white to a rather vivid green. In the ninth fuzzy-seeded segregate all of the seeds had uplandlike fuzz (fig. 8, E), ranging in degree from half covered to completely covered.

The fact that the fuzzy-seeded segregates in  $F_2$  of combination 8 resembled upland cotton rather than Pima in the quantity and length of the fuzz on the seeds points to the conclusion that the Ballard parent of this cross, while probably homozygous for the major factor which determines smooth seededness as contrasted with fuzzy seededness, also carried an intensifier for fuzziness which could express itself in  $F_2$  only in those segregates which possessed the major factor for fuzziness contributed by the Pima parent of the combination.

Combination 9 (Table 4) represents the interspecific hybrid Egyptian  $\times$  upland in which the Egyptian (Pima) parent belonged to the homozygous smooth-seeded family which furnished one of the parents of the crosses ( $A \times H$ , etc.) described in the earlier pages of this paper, and of combinations 1 and 2 in Table 4; and the upland parent belonged to a typical family of the very fuzzy-seeded variety Lone Star. This combination affords the only indication of dominance of the fuzzy-seeded condition over the smooth-seeded condition obtained in this investigation. In the two  $F_1$  progenies of 10 plants each, all were fuzzy seeded.<sup>14</sup> The segregation in  $F_2$  was less clear cut than in the other combinations, but dominance of the fuzzy-seeded condition is strongly indicated, since 11 of the 14 plants had fuzzy seeds. Disregarding one plant of doubtful classification, the value for  $\chi^2$  of the departure of the observed distribution in  $F_2$  from that expected with a ratio of 1 smooth to 3 fuzzy, is 0.64, indicating a chance of about 1 in 2.5 that the departure is fortuitous.

In both  $F_1$  progenies many of the plants had seeds that were only partly covered with fuzz. Of the fuzzy-seeded segregates in  $F_2$ , six had the seeds completely covered (fig. 9, F), but there was considerable plant-to-plant variation in the length of the fuzz and in its color (whitish, brown, or green). Another  $F_2$  plant had most of the seeds completely covered and the rest almost completely covered (fig. 9, E); whereas the remaining four fuzzy-seeded segregates ranged in degree of fuzziness from grade 5 to grade 9 of the Pima scale (fig. 9, C and D). The two  $F_2$  plants classed as smooth seeded had somewhat more fuzz on the seeds than the smooth-seeded Pima family, corresponding to grade 2 of the Pima scale. (Fig. 9, A.) One plant in this  $F_2$  progeny showed an anomalous condition. About half of the surface of the seed coat was sparsely covered with hairs which resembled short lint hairs, but there was a minute tuft of typical fuzz at the small end of the seed. (Fig. 9, B.) This plant probably belongs to the smooth-seeded class.

A comparison of the results from combinations 3 and 9 (Table 4) is particularly interesting. In combination 3 a phenotypically smooth-seeded and probably homozygous upland plant was crossed with a homozygous recessive (fuzzy-seeded) upland plant, while in combination 9 a homozygous smooth-seeded Egyptian plant was crossed

<sup>14</sup> This result is not in accord with that noted by Thadani (13), who states that in a cross of the comparatively smooth-seeded Yuma variety of Egyptian cotton with a fuzzy-seeded upland, the smoother seeded condition was dominant in  $F_1$ .

with a member of the same homozygous recessive upland family which furnished the fuzzy-seeded parent of combination 3. Dominance of the smooth-seeded condition is shown in combination 3 and domi-

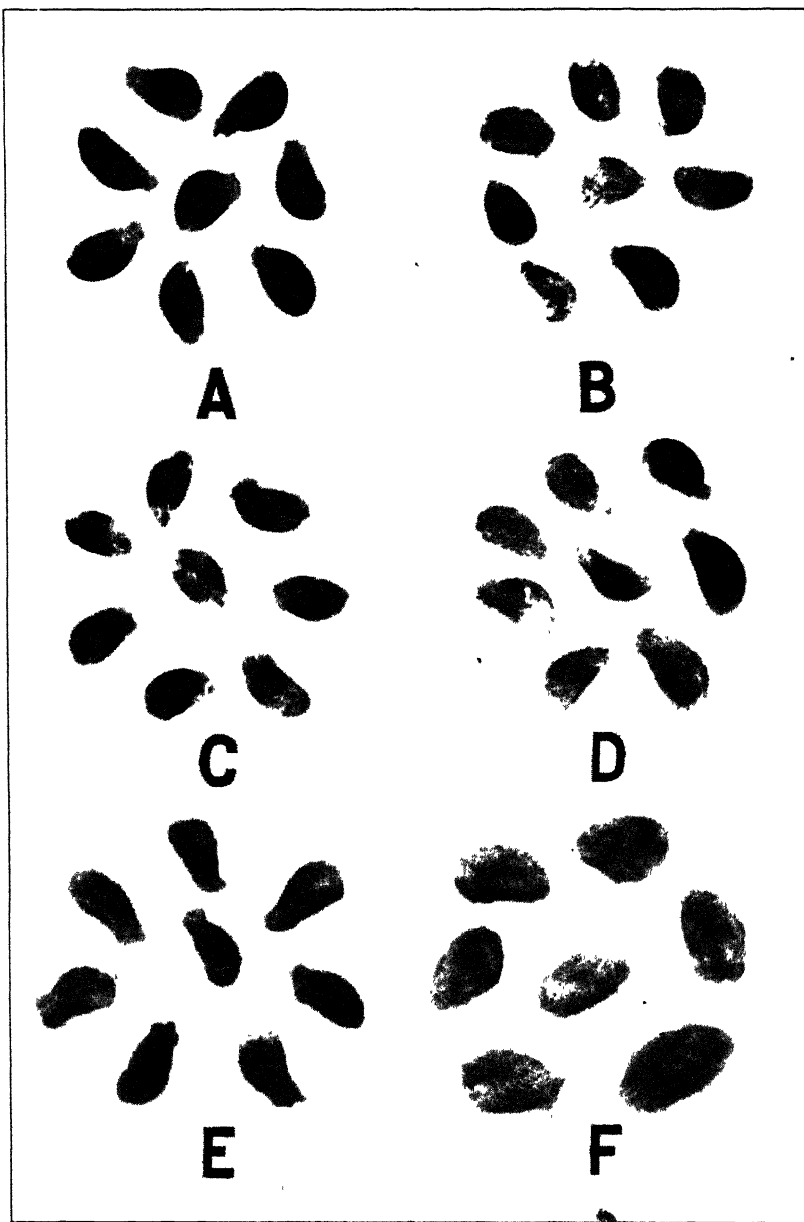


FIG. 9.—Seeds of  $F_2$  plants of combination 9, a cross between the smooth-seeded Pima family (P-SS-7) and a fuzzy-seeded upland family (Lone Star), showing the several types of segregates: A, having localized fuzz, as in the Pima family, and classed as smooth seeded; B, nearly fuzzless, most of the hairs being adherent lint hairs; C and D, Pimalike in the character and distribution of the fuzz (classed as fuzzy seeded); E, completely or almost completely fuzzy seeded, fuzz short and sparse; F, completely fuzzy seeded, fuzz long and dense

nance of the fuzzy-seeded condition is shown in combination 9. Since there is no reason to doubt that the individual fuzzy-seeded parents of the two combinations were genetically identical in respect to this character, it would seem probable that the Ballard naked-seeded family of upland cotton and the smooth-seeded family of Egyptian (Pima) cotton have different factors for smooth seeds, the upland factor being dominant and the Pima factor recessive to the fuzzy-seed factor in Lone Star upland. This seems the less surprising in view of the fact that in the smooth-seeded upland family the fuzz normally is entirely wanting or reduced to a very slight trace (fig. 2), while in the smooth-seeded Pima family a pronounced though sharply localized tuft of fuzz is almost invariably present (fig. 1, A).

The conclusion that the factors for smooth seed in the two families are different might be regarded as weakened by the behavior of the cross between them (combination 7), since the uniformly smooth-seeded condition in  $F_1$  suggests that the Egyptian and the upland parent had the same major factor for smooth seededness. On the other hand, the fact that 85 per cent of the smooth-seeded segregates in  $F_2$  resembled the upland rather than the Egyptian parent in their naked or very nearly naked seeds, strengthens the assumption that the smooth-seeded forms of the two types of cotton have a different factorial constitution.

The factor for smooth seeds in Pima, although recessive to the factor for fuzzy seeds in Lone Star upland, is dominant to the factor for fuzzy seeds in Pima, as is shown clearly by the results with combinations  $A \times H$ , etc., described in the earlier pages of this paper, and with combinations 1 and 2. (Table 4.) It may be recalled in this connection that the degree of fuzziness in the fuzzy-seeded Pima families (fig. 1, B and C), is always much less than in the Lone Star upland family (fig. 3, A).

It can not be determined from the results with combinations 4, 5, and 6 (Table 4) whether the fuzzy-seeded Pima and upland families differ in the major factor for seed fuzziness, although it is almost certain that the two types of cotton possess different modifying factors for this character. This supposition is favored by the occurrence of typical upland fuzz in some of the  $F_2$  segregates of a cross between a fuzzy-seeded Egyptian plant and a presumably homozygous smooth-seeded upland plant (combination 8); and also by the fact that in crosses between a fuzzy-seeded Egyptian and a fuzzy-seeded upland (combinations 4, 5, and 6) many of the  $F_2$  segregates exceeded the fuzzier seeded (upland) parent in the length and density of the fuzz. It also seems likely that the fuzzy-seeded upland parental families differ genetically. This is indicated by a comparison of the fuzziest seeded segregates of combinations 4 and 5. The same homozygous Egyptian (Pima) family furnished one of the parents of these combinations, but the upland parent was of the Acala variety in combination 4 and of the Holdon variety in combination 5. Holdon (fig. 3, C) has much longer and denser fuzz than Acala (fig. 3, B), and some of the  $F_2$  segregates of combination 5 (fig. 5, D) had much longer and denser fuzz than the fuzziest seeded  $F_2$  segregates of combination 4 (fig. 5, B).

## LINKAGE RELATIONS

## SEED FUZZINESS AND PETAL SPOT

The combinations described in the earlier pages of this paper ( $A \times H$ , etc.) had as the fuzzy-seeded parents members of a Pima family characterized by almost complete absence of the large dark red petal spot which is of almost universal occurrence in the Egyptian type of cotton. The "spotless" condition has been shown to be recessive to normal development of the spot and to be determined by a single major factor (9). It will be of interest to note the inheritance of this character in relation to that of smooth seededness in the combinations dealt with in this paper.

The  $F_1$  of combinations  $A \times H$ , etc., grown in 1924, had the spot present on all plants, although it was less developed than in the normally spotted parent. This was in accordance with the results of previous observation (9, p. 494). Likewise, as in the earlier investigation (9, p. 496), the incompleteness of the dominance of spotted petal was shown in  $F_2$ . The dominance is so nearly complete, however, that separate classification of the homozygotes and heterozygotes of the spotted class is very difficult and was not attempted in considering the results in  $F_2$ .

Since spotless petal is recessive to the spotted condition and fuzzy seededness is recessive to smooth seededness,  $F_2$  should exhibit a 9:3:3:1 ratio provided there is no linkage of the two genes and no differential survival among the several classes of gametes and zygotes.

In determining whether the two characters are linked, the procedure was as follows: The observed percentage of spotless individuals in  $F_2$  having been 19.4 and the observed percentage of fuzzy-seeded individuals having been 27.6, the percentage of the spotless-fuzzy (double-recessive) class should have been 19.4 per cent of 27.6 per cent = 5.35 per cent. The expected percentages for the other classes, computed on the same basis, are: Spotted smooth (double dominant), 58.35 per cent; spotted fuzzy, 22.25 per cent; and spotless smooth, 14.05 per cent. The expected numbers in the several classes, corresponding to the expected percentages, as compared with the observed numbers, are shown in Table 5. The value for  $\chi^2$  (0.1628) indicates a very good fit, the chances being about 7 in 10 that the departure from the expectation is fortuitous. It may be concluded, therefore, that there is no linkage between the genes for presence of the petal spot and smoothness of the seed coat.

On the assumption of a 9:3:3:1 ratio, which would make the expected numbers for the several classes 214.3, 71.4, 71.4, and 23.8,  $\chi^2$  for the departure of the observed distribution is 8, indicating a chance of only 1 in 200 that the departure from the expectation is fortuitous. It is probable, therefore, that there was a small degree of differential survival of gametes or of zygotes, favoring the class with spotted petals at the expense of the spotless class. For the departure from the expectation in this case (three spotted to one spotless),  $\chi^2$  is 6.3, indicating a chance of only about 1 in 100 that the deficit of spotless individuals is fortuitous. There is also a smaller deficit of smooth-seeded individuals, but this departure probably is fortuitous,  $\chi^2$  being only 1.3.

TABLE 5.—Percentages and numbers expected in the absence of linkage and numbers observed in the several classes in  $F_2$  of crosses between a spotted smooth-seeded and a spotless fuzzy-seeded family of Pima cotton

[Combinations  $A \times H$ ,  $A \times J$ ,  $B \times H$ ,  $B \times J$  as one array]

Class	Percentage expected	Number	
		Expected	Observed
Spotted smooth.....	58.35	222 3	221
Spotted fuzzy.....	22.25	84 8	86
Spotless smooth.....	14.05	53.5	55
Spotless fuzzy.....	5.35	20 4	19
Total.....	100	381	381

#### SEED FUZZINESS AND ABUNDANCE OF LINT

Working with "American cotton," presumably upland, and crossing a smooth-seeded (fuzzless) form having sparse lint with a

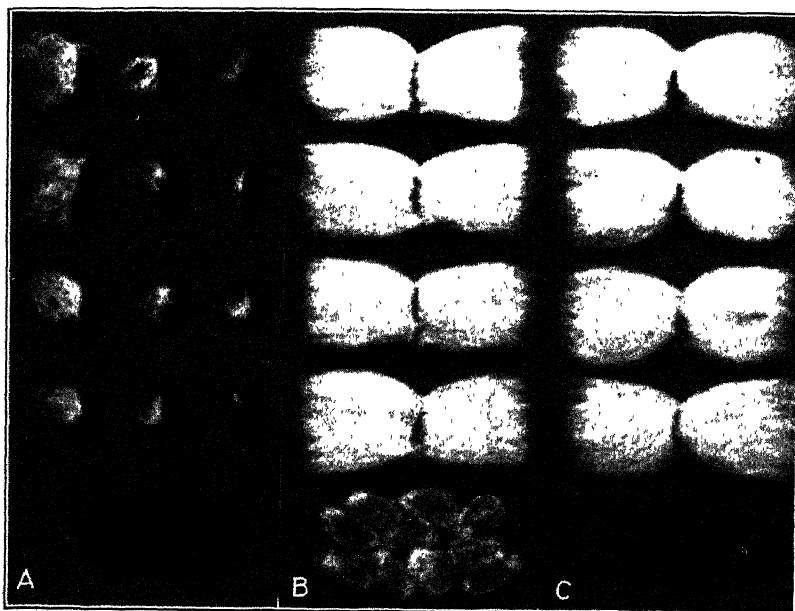


FIG. 10.—Seeds, before and after removal of the lint hairs, showing the dominance of smooth seeds and abundant lint in a cross between two upland cottons made by W. W. Ballard: A, the parent which had smooth seeds and sparse lint; B, the parent which had fuzzy seeds and abundant lint; C,  $F_1$  which had smooth seeds and abundant lint

fuzzy-seeded form having abundant lint, Thadani (12) found smooth seeds and abundant lint to be dominant in  $F_1$ . In  $F_2$  either character separately gave a near approach to a 3:1 ratio, whereas the distribution with respect to both characters was as follows: Smooth seeds and abundant lint, 103; smooth seeds and sparse lint, 54; fuzzy seeds and abundant lint, 53; fuzzy seeds and sparse lint, none. Thadani concluded that there is complete linkage of smooth seeds and sparse lint.

Similar crosses of two upland cottons were made in 1921 at Greenville, Tex., by W. W. Ballard, to whom the writers are indebted for use of the resulting data. The two parent individuals having smooth seeds and sparse lint were of the same Ballard family which furnished one of the parents of combinations 3, 7, and 8 described in this paper. The two parent individuals having fuzzy seeds and abundant lint were of the Lone Star variety. Both  $F_1$  progenies showed complete dominance of smooth seeds and of abundant lint. Seeds, with and without lint, of the parents and of  $F_1$  are shown in Figure 10. From selfed seed of an individual in each  $F_1$  progeny, an  $F_2$  progeny was grown at Greenville in 1923, the  $F_2$  populations having comprised, respectively, 164 and 100 plants. As classified by Ballard, the distributions in  $F_2$  were strikingly similar to the one recorded by Thadani. In this case also either character separately gave a near approach to a 3:1 ratio, and the double-recessive class was not represented.

In order to determine whether there is linkage of the two characters, the same procedure was followed as in dealing with seed fuzziness and petal spot. The percentages and numbers expected in case there were no linkages and the numbers observed in  $F_2$  are shown in Table 6. Since  $\chi^2$  of the departure of the observed from the expected distribution amounts to 17.2 in progeny A and 8.2 in progeny B, the chances are very small that the departures are fortuitous; and it may be concluded that linkage of smooth seeds and sparse lint is complete or nearly so, as was the case in Thadani's material. Whether there was crossing over, and if so, in what proportion, can not be determined with certainty, because of the relatively small size of the  $F_2$  populations and the fact that the double-recessive class was not represented.

TABLE 6.—Percentages and numbers expected in the absence of linkage and numbers observed in the several classes in  $F_2$  of crosses made by Ballard between a smooth-seeded sparse-linted and a fuzzy-seeded abundant-linted family of upland cotton

Class	Progeny A			Progeny B		
	Percentage expected	Number		Percentage expected	Number	
		Expected	Observed		Expected	Observed
Smooth abundant.....	57.2	93.8	84	59.9	59.9	55
Smooth sparse.....	19.0	31.2	41	13.1	13.1	18
Fuzzy abundant.....	17.8	29.2	39	22.1	22.1	27
Fuzzy sparse.....	6.0	9.8	0	4.9	4.9	0
Total.....	100	164	164	100	100	100

### SUMMARY

It is desirable that the seeds of long-staple cottons which are ginned on the roller type of gin should be as free as possible from fuzz or short hairs. This makes smooth seededness an important objective in breeding work. Knowledge of the manner of inheritance of smooth and fuzzy seeds will therefore be useful to cotton breeders.

This paper presents the evidence, from crosses between smooth-seeded and fuzzy-seeded cottons, that the inheritance of this character is mainly of a simple Mendelian type.

Crosses were made between representatives of a family of Pima Egyptian cotton homozygous for very little fuzz on the seeds, designated the smooth-seeded Pima family, and representatives of a family homozygous for the much more fuzzy condition of the seed coat typical of the same variety.  $F_1$  was uniformly smooth seeded and the segregation in  $F_2$  was in the ratio 3 smooth to 1 fuzzy. The smooth-seeded and the fuzzy-seeded segregates closely resembled the respective parents.

Third-generation progenies were grown from self-pollinated seed of 20 smooth-seeded and of 10 fuzzy-seeded  $F_2$  plants. The progenies of fuzzy-seeded  $F_2$ 's were uniformly fuzzy seeded. Of the smooth-seeded  $F_2$  plants, two were probably homozygous, their  $F_3$  progenies having contained no fuzzy-seeded plants. The remaining 18 progenies showed segregation in various proportions, but when taken as one array, 24.6 per cent of the population were fuzzy seeded, and the departure from the expectation (25 per cent) was only one-fourth its probable error. The evidence justifies the conclusion that in the Pima variety the fuzzy condition is recessive to the smooth condition.

The same and other smooth-seeded and fuzzy-seeded families also were used in another series of combinations. (Table 4.) Some of these were hybrids between very different species of *Gossypium*, Egyptian cotton (*G. barbadense*  $\times$  ?) and upland cotton (*G. hirsutum*). The hybrid populations of these combinations were small, and the results obtained are to be regarded as suggesting rather than proving the nature of the inheritance.

Of the families which furnished the parents of these combinations, the smooth-seeded family of upland cotton had the seeds almost entirely devoid of fuzz, hence much smoother than in the smooth-seeded family of Egyptian (Pima) cotton. The fuzzy-seeded families of upland cotton, on the other hand, had much fuzzier seeds than the fuzzy-seeded families of Egyptian (Pima) cotton.

Crosses (combinations 1 and 2) between the smooth-seeded Egyptian (Pima) family used in the first series of combinations and another fuzzy-seeded Egyptian (Pima) family, not related to the family used in the first series, showed similar dominance of smooth-seededness and monohybrid segregation in  $F_2$ .

The results of a cross between two plants of upland cotton, one phenotypically smooth seeded and probably homozygous, the other homozygous for fuzzy seeds (combination 3), indicated that in upland cotton also there is dominance of the smooth condition, and monohybrid segregation in  $F_2$ .

Crosses between fuzzy-seeded Egyptian (Pima) and fuzzy-seeded upland families (combinations 4, 5, and 6) produced only fuzzy-seeded plants in  $F_1$  and, almost certainly, only fuzzy-seeded plants in  $F_2$ , the results indicating that all of these families were homozygous.

A cross between a homozygous smooth-seeded Egyptian (Pima) plant and a phenotypically smooth-seeded upland plant (combination 7) gave a uniformly smooth-seeded  $F_1$  and a near approach to a 3 (smooth) to 1 (fuzzy) ratio in  $F_2$ . The occurrence in  $F_2$  of fuzzy-seeded segregates which resembled upland cotton in the quantity and character of the fuzz, suggests that the individual of the

smooth-seeded upland family which served as one of the parents of this cross was heterozygous.

A cross between a homozygous fuzzy-seeded Egyptian (Pima) plant and a phenotypically smooth-seeded upland plant (combination 8) gave a uniformly smooth-seeded  $F_1$  and segregation in  $F_2$  in the proportion of 12 smooth to 9 fuzzy, which, because of the small size of the population, is probably not a significant departure from a 3:1 ratio. Although the absence of fuzzy seeds in  $F_1$  indicates that the upland parent was homozygous in respect to the major factor for smooth seeds, yet this plant may have carried an intensifier for fuzziness, since the fuzzy-seeded segregates in  $F_2$  had the upland degree of fuzziness.

A cross between a homozygous smooth-seeded Egyptian (Pima) plant and a homozygous fuzzy-seeded upland plant (combination 9) gave only fuzzy-seeded plants in  $F_1$  and segregation in  $F_2$  in approximately the ratio of 1 smooth to 3 fuzzy. In both hybrid generations there was considerably greater variation in the quantity and character of the fuzz than was the case with the other combinations.

The behavior of combination 9 indicates dominance of the upland factor for fuzzy seeds over the Egyptian factor for smooth seeds, whereas in combination 3 the upland factor for fuzzy seeds was recessive to the upland factor for smooth seeds. Since the same (doubtless homozygous) upland family furnished the fuzzy-seeded parents of both combination, it may be concluded that different factors for smooth seeds occur in Egyptian and in upland cotton.

It also seems probable that the slightly fuzzy condition of the smooth-seeded Egyptian is recessive to the naked or nearly naked condition of the smooth-seeded upland, since in the cross between a smooth-seeded Egyptian and a smooth-seeded upland (combination 7) 85 per cent of the smooth-seeded segregates in  $F_2$  had the seeds naked or very nearly so.

The factor for smooth seeds in Egyptian (Pima), although recessive to the factor for fuzzy seeds in upland, is dominant to the factor for fuzzy seeds in Egyptian (Pima), as was shown in the earlier pages of this paper.

The occurrence of modifying factors or intensifiers for fuzziness is indicated by the results from crosses of fuzzy Pima with fuzzy upland (combinations 4, 5, and 6). That such a factor or factors may be carried by a plant homozygous for the major factor determining smoothness as contrasted with fuzziness is suggested by the results of the cross between fuzzy Pima and smooth upland (combination 8).

The crosses described in the early pages of this paper had for their parents members of a Pima Egyptian family homozygous for both smooth seeds and normally spotted petals and members of another Pima family homozygous for both fuzzy seeds and almost spotless petals. Complete independence of the characters, smooth and spotted, is demonstrated by the results from these crosses.

Crosses made by Ballard between members of a family of upland cotton having fuzzless seeds and sparse lint and members of an upland family having entirely fuzzy seeds and abundant lint gave satisfactory evidence of complete or nearly complete linkage of fuzzless seeds and sparse lint. The results confirm those which Thadani obtained with a similar cross.

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# THE PRODUCTION OF CERTAIN ENZYMES BY *BACTERIUM PRUNI*<sup>1</sup>

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## INTRODUCTION

Erwin F. Smith (8, 9, 10, p. 57-60)<sup>2</sup> observed that *Bacterium pruni*, studied and named by him, forms characteristic crystals when grown aerobically in skim milk. Upon request of Doctor Smith, the writer made a biochemical study of this disease-producing organism parasitic on plum and peach trees (6, 8, 9, 10, p. 57-60). The study led to the identification of the aforementioned crystals, which were shown to be made up of tyrosine, leucine, and higher fatty acids (4). Of these compounds tyrosine was identified by the xanthoprotein, Millon, and Mörner reactions and the melting point and nitrogen estimations, while leucine was identified by the form of its crystals, its ability to sublime, and its melting point. Certain properties of the third constituent of the crystals, namely, the light specific gravity, the ability to form a fatty spot on paper, and the melting point, have shown it to be a mixture of stearic, palmitic, and myristic acids. These were demonstrated to be present partly in the free state and partly in the form of a calcium salt, as shown by the ability of the latter to form, on distillation, the corresponding ketones, stearone, palmitone, and myristone.

## PROTEOLYTIC AND LIPOLYTIC ENZYMES FOUND

While the primary object of identifying the crystals had thus been attained, it was realized that a consideration of the enzymes produced by the microbe would throw additional light on its metabolism. Because of the occurrence in milk of the proteins casein, lactoglobulin, lactalbumin, and opalisin (1, p. 383; 5; 7), attention was naturally directed first to the proteolytic enzymes. The proteases are known to have the ability to digest and to split up proteins of plant and animal origin into amino acids (2, 11). This was exactly the case when *Bacterium pruni* was grown in milk. Despite the fact that growth of the organism occurred at room temperature, and not at body temperature (37-38° C.), generally considered to be the optimum temperature for the activities of enzymes (3, p. 521), the amino acids tyrosine and leucine have definitely been shown to result in the metabolism of the milk proteins by *Bact. pruni*. That tyrosine and leucine, the well-known constituents of casein, lactoglobulin, and lactalbumin, were shown to be among the products of growth of the microbe can not be surprising when it is considered that they are difficultly soluble amino acids. That other amino acids which may

<sup>1</sup> Received for publication May 2, 1927; issued September, 1927.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 220.

have been present were not in evidence, was probably due to their greater solubility in the culture medium employed and perhaps also to the fact that because of their ready solubility they could be utilized by *Bact. pruni* for the structural process (building up its tissues).

The occurrence of butterfat in milk directed attention to the possible presence of lipolytic enzymes in the culture medium. Lipases are known to split fats into their component parts, glycerol and fatty acids (2, p. 237). This was true of the culture medium in which *Bact. pruni* was grown. Stearic, palmitic, and myristic acids were shown to be present in it. That the lower fatty acids, such as butyric, caproic, and caprylic, were not present in the crystals was probably due to their comparatively greater solubility in the nutrient medium. That a part of the identified acids referred to above has been found in the form of a calcium salt can not be surprising in view of the fact that calcium is known to be a normal constituent of milk.

Mention should be made of the fact that the skim milk in which *Bacterium pruni* was grown had been sterilized on four consecutive days for fifteen minutes each, at a temperature of 100° C. This heating of the milk, which practically has a neutral reaction, could not possibly have any disintegrating effect upon its native proteins. The latter, it will be remembered, must be boiled for several hours with *strong acids* or *alkalies* before they can be split up into amino acids. Nor could the butterfat undergo hydrolysis under the conditions mentioned. Furthermore, the heated milk was perfectly free from enzymes which, while present in the original milk, were completely destroyed by the process of sterilization. Consequently, the amino acids and fatty acids found in the milk must necessarily be ascribed to the action of the enzymes which were elaborated by the microbe upon the milk constituents.

### SUMMARY

Proteolytic and lipolytic enzymes have been shown to be present among the products of growth of *Bacterium pruni* in milk.

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# INHERITANCE OF WINTER HARDINESS AND GROWTH HABIT IN CROSSES OF MARQUIS WITH MINHARDI AND MINTURKI WHEATS<sup>1</sup>

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## INTRODUCTION

Winter wheat usually matures earlier than spring wheat and thus often escapes the hot, dry winds and rust which frequently injure spring wheat. When winterkilling is not a factor, the yield of winter wheat is greater, as a rule, than that of spring wheat. Winter wheat has the advantage of aiding in the distribution of labor upon the farm. For these reasons it is generally grown instead of spring wheat wherever it proves successful.

The introduction of the hard red winter wheats into this country has resulted in a northward expansion of the area devoted to the culture of winter wheat. Low temperatures, combined with other environmental factors to which winter wheat often succumbs, have limited its wider use. The acreage of winter wheat abandoned annually in the United States, primarily as the result of winterkilling, varied from 1.1 to 28.9 per cent in the 25-year period from 1901 to 1925, inclusive. The average abandonment for the five years from 1921 to 1925 was 12.5 per cent, and in 1925 the estimated percentage was 21.7 (1).<sup>2</sup>

The development of more hardy varieties has made possible the successful growing of winter wheat in those areas where formerly it frequently was injured by winterkilling. As an illustration may be mentioned the variety Minturki, which was obtained by crossing Odessa, a late-maturing, winter-hardy variety, with Turkey. The new variety has proved very successful in southern Minnesota.

In addition to the inability of fall-sown wheat to survive the winter in the upper Mississippi Valley, the hard red winter wheats have not measured up to the high standards of quality of grain which are characteristic of the better spring wheats. Marquis is undoubtedly the chief spring-wheat variety which is responsible for this difference. It is natural, therefore, that there should be a demand for winter wheats of better quality and greater winter hardiness than those varieties now being grown in this area. This paper deals with the inheritance of these important characters in crosses between spring

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<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 235.

and winter wheats and with the production of improved strains of hard red winter wheats which will withstand the severe winters of the North-Central States.

#### PREVIOUS STUDIES OF WINTER AND SPRING WHEAT TYPES AND INHERITANCE OF DIFFERENTIAL CHARACTERS

Linnaeus (15), in his "Species Plantarum," 1753, classified wheat as spring (*Triticum aestivum*) and winter (*T. hybernium*). Villars (21), in 1787, combined the two groups into *T. vulgare*. Clark, Martin, and Ball (6) classified varieties into the two classes, and other investigators (8, 10) placed the wheats in three general groups according to their manner of growth, as follows:

- (a) Winter-growing habit—those which, when sown in the spring, fail to head.
- (b) Spring-growing habit—those which fail to survive the winter when sown in the fall in the more northerly regions but head normally from spring sowing.
- (c) Intermediate forms—those which are hardy enough to survive the winter when fall sown and also mature as spring wheats when spring sown.

All fall-sown wheat varieties grown in the Great Plains area of the United States have the true winter habit. Most, if not all, of the wheats sown in the fall or winter in California and Arizona are true spring wheats.

Takahashi (19) classified barley varieties on the basis of growth habit and winter hardiness, as follows:

- (a) Winter forms, which are winter hardy but fail to ripen when spring sown.
- (b) Spring forms, which are killed when planted in the fall.
- (c) Intermediate forms, which are winter hardy and also ripen when sown in the spring.
- (d) Pseudowinter forms, which have the winter habit from spring sowing but are not winter hardy.

The first three groups in this classification of barley varieties correspond to those of wheat. The fourth class (pseudowinter) of the barleys undoubtedly comprises forms with the winter habit of growth which are not winter hardy. A similar class found in wheat is mentioned in this paper.

Fruwirth (10), Killer (14), Waldron (22), and Vavilov and Kouznetsov (20) demonstrated that spring types could not be selected from pure lines of winter wheats. The selection of spring types from winter wheats was successful only in those cases where both types were present before the selections were made. Fruwirth was not able to alter the growth habit of pure-line selections by growing these out of season for several generations. A single head of wheat was divided into two parts, and the seed from one-half was sown in the fall and that from the other half in the spring. The selections were grown from fall and spring sowing for eight years and then both lots were sown together in the two seasons. The growth habit and periods of blossoming and ripening of the two lots were found to be the same.

There are numerous reports in the literature on the relative resistance of wheat varieties to killing by low temperatures. There are relatively few reports, however, on the inheritance of cold resistance. Nilsson-Ehle (16), in Sweden, as early as 1900, began studies of the behavior of different varieties of winter wheat and their hybrids under various degrees of winter severity. Twelve years later he reported the results obtained from a cross between two medium-

hardy varieties of winter wheat. From this cross, lines were produced which extended beyond the limits of the parents in both directions. He concludes that the winter resistance character is inherited in the same manner as other quantitative characters and is controlled by several Mendelian factors.

Hayes and Garber (13), in Minnesota, report the production of winter wheats which were more resistant to injury by low temperatures than either of the parents. Two of these varieties, Minhardi and Minturki, were produced by crossing Turkey and Odessa. Odessa is a very winter-hardy variety which was obtained from Russia. Both Minturki and Minhardi have proved very winter hardy in Minnesota. They have proved even more winter hardy than the more hardy parent, Odessa, as has been demonstrated by Clark, Martin, and Parker (?), who have made a comparative test of the hardiness of winter-wheat varieties for a period of six years (1920-1925) over a wide area in the Northern States and adjacent Canada.

From crosses between two varieties of intermediate cold resistance, Åkerman (3), in Sweden, obtained hybrids, some of which were superior and others inferior to the parents. Most of the hybrids had an intermediate degree of cold resistance.

Schafer (17), in a brief report of inheritance studies at the Washington Agricultural Experiment Station, states that hardiness was recessive to nonhardiness in a cross between Turkey (winter) and Jenkin (club, spring) wheats. A majority of the  $F_3$  rows showed severe winter injury.

Martin,<sup>3</sup> after a study of the inheritance of cold resistance in crosses between Kanred and Minhardi, concludes that it is difficult to study cold resistance without controlling temperatures. A large number of crosses of bulk  $F_3$  generations were studied which were intermediate in winter hardiness as compared with their parents. Martin<sup>4</sup> and Vassar<sup>5</sup> both obtained a low correlation between the survival of winter-wheat hybrid lines in certain of the segregating generations and the progeny of individually selected plants from these lines in the following generation.

In 1918-19, 75  $F_1$  plants from a cross between Marquis and Kanred were grown in the plant-breeding nursery at St. Paul, Minn. The fact that 73 of these plants survived the winter would seem to indicate that hardiness of the winter parent was dominant or partially so. The later generations were all grown from spring seeding, and consequently no further studies of this character were made.

Detailed reviews have been published of the inheritance of growth habit in crosses between spring and winter cereals (2, 8, 11, 12). Only a brief summary will be given here. Two investigators, Spillman (18) and Fruwirth (9), report a dominance of the winter over the spring habit of growth. Caporn (5) found the  $F_1$  intermediate in time of ripening. Vavilov and Kouznetsov (20), Aamodt (2), and Gaines and Singleton (12) report a dominance of the spring over the winter habit of growth and conclude that the inheritance of this character is controlled by multiple factors. Cooper (8)

<sup>3</sup> MARTIN, J. H. COMPARATIVE STUDIES OF WINTER HARDINESS IN WHEAT. [In press.]

<sup>4</sup> MARTIN, J. H. Op. cit.

<sup>5</sup> VASSAR, L. P. FIELD AND LABORATORY METHODS FOR A DETERMINATION OF WINTER HARDINESS IN WINTER-WHEAT HYBRIDS. [Unpublished thesis, Univ. Minn.]

likewise reports a dominance of the spring over the winter habit, but explains his 13:3  $F_2$  ratio with a dominant factor for winter habit and an inhibiting factor of winter habit. The results reported by Cooper from the  $F_3$  do not satisfactorily support the factorial hypothesis advanced for the  $F_2$ .

#### MATERIALS AND EXPERIMENTAL METHODS

The principal varieties of wheat used in this study are Marquis, Minhardi, and Minturki. Marquis, a high-yielding, high-quality, hard red spring wheat, was used as the spring parent in crosses with Minhardi and Minturki as the winter parents. Marquis is reported by Martin<sup>6</sup> as being one of the hardiest known spring wheats when sown in the fall. Cooper (8), at Ithaca, N. Y., found that the percentage of winterkilling in fall-sown Marquis was not great. At St. Paul, Minn., (4) fall-sown Marquis has not been known to live through the winter. In the experiments reported in this paper it was planted as a check in about every eleventh row throughout the nurseries.

The varieties used as the winter parents are two of the hardiest known winter wheats (7, 13). Minhardi shows the highest average winter survival of all varieties grown in the extensive hardiness trials conducted by Clark, Martin, and Parker (7). Minturki in these same trials had the third highest percentage of survival. Both of these wheats are selections from a cross made at University Farm, St. Paul, Minn., in 1902, between Odessa (Minn. Accession No. 558) and Turkey (Minn. Accession No. 829) (7). Minturki, while not as winter hardy as Minhardi, is superior in yield, quality, and resistance to attacks of stem rust and is nearly the equal of Minhardi in cold resistance.

The plants were spaced 3 inches apart in the row and the rows were 1 foot apart. The  $F_1$  plants were grown from both fall-sown and spring-sown seed. The  $F_2$  from fall sowing were grown in two plots at some distance from each other, and killing was more severe in one location than in the other. In  $F_3$  and  $F_4$  in the winter-wheat nursery, each hybrid line for which seed was available was grown in four systematically distributed rows, each row containing 25 seeds. The parents were grown as checks in a similar manner about every eleventh row.

In order to average results from separate rows, a hardiness index figure was computed on the basis of 100 as representing a perfect stand of vigorous plants. The numbers of strong, weak, and dead plants were determined in the spring for each replication of each line. To obtain a figure for each row, the strong plants were given a value of 4, the weak 2, and the dead 0. The results were then calculated on a 25-plant basis, as there was not a full stand in all of the rows. The hardiness figures presented are averages of the hardiness of the separate trials.

In the  $F_2$  the plants which had survived the winter were selected for growing in the  $F_3$ . Selections were made for early, medium, and late maturity. Others were selected on the basis of condition of the plant in the spring. Those that were large and vigorous in the spring were recorded as "strong," while those which barely survived were

<sup>6</sup> MARTIN, J. H. Op. cit.

recorded as "weak." In another experiment only vigorous plants were selected at random.

Numerous references and citations could be made regarding the physiological studies on the nature of cold resistance in wheat and many other plants. Martin<sup>7</sup> has recently made a comprehensive study of the literature and the methods employed to determine the hardiness of a given variety of winter wheat. He states that "most characters suggested for a test of winter hardiness, if dependable, hold for only a limited number of varieties or limited set of conditions." Studies of the inheritance of winter hardiness often are unsatisfactory because the winter may be too severe and may eliminate all or most all of the hybrid material, or it may be less severe and all of the plants may survive. The writers were fortunate in being so situated that the studies could be carried on under borderline conditions for killing. At University Farm, St. Paul, Minn., there is a rather consistent elimination of the less hardy plants and varieties and a survival of the hardier ones. In this study the determinations of winter hardiness were made in the field under conditions whereby the spring-wheat parent was eliminated when fall sown and the winter parent had a fairly high survival.

When seed was available, progeny of the same  $F_2$  and  $F_3$  plants which survived the previous winter were grown also as spring wheats. In the spring sowings the date of emergence of the main spike of each plant was noted and all of the plants were placed in groups by weekly heading periods. One week from the day on which the first plant headed, tags were placed on plants on which one or more spikes had emerged. These comprised the first class. One week later the plants which had headed after the first period were tagged. These comprised the second class. This process was followed until the end of the season, when no more plants headed. There were seven classes for heading periods and one class for nonheading, which comprised the true winter plants.

Following the selection of the plants in the field for vigorous growth, they were threshed individually and a determination of the seed quality was made in the laboratory. The seed of each plant was examined for hardness, texture, and color. Those which were hard, vitreous, and dark red were selected for growing in the following generation. If the seed of all of the plant selections from any line appeared to be low in quality, the seed of at least one plant of that line was sown, in order to study the inheritance of its characters in the following generation.

## EXPERIMENTAL RESULTS

### STUDIES OF $F_1$ AND $F_2$

The  $F_1$  plants which furnished seed for the  $F_2$  were grown in the spring of 1923. They headed approximately as early as the Marquis parent, while the winter-wheat parents, Minhardi and Minturki, when sown in the same spring, failed to head. The  $F_1$  was also sown in the fall of the same year, in order to test its ability to survive the winter.  $F_2$  generations, as well as the parental varieties, Marquis and Minhardi, were sown in the fall of 1923. None of the seed from

<sup>7</sup> MARTIN, J. H. Op. cit.

the  $F_1$  plants was saved for spring sowing, as the primary purpose was to study the inheritance of cold resistance in relation to other characters which differentiated winter and spring wheats.

The results of these studies are given in Table 1. The winter was not particularly severe, and the Minhardi parent survived, 42 plants being classed as strong and 1 as weak. The  $F_1$  plants were all winter-killed. It will be remembered that the  $F_1$  headed practically as early as Marquis when sown in the spring. Apparently spring habit of growth is dominant over winter habit, and cold resistance behaves as a recessive character. From 33 to 55 per cent of the  $F_2$  plants survived.

TABLE 1.—A comparison of the survival of Marquis, Minhardi, and  $F_1$  and  $F_2$  generations of Marquis  $\times$  Minhardi and Marquis  $\times$  Minturki wheats at University Farm, St. Paul, Minn., in the winter of 1923-24

Variety or cross	Total plants	Classified as—			Per-centage of sur-vival	Hardi-ness index
		Strong	Weak	Dead		
Marquis.....	100	0	0	100	0	0
Minhardi.....	43	42	1	0	100	99
Marquis $\times$ Minhardi, $F_1$ .....	12	0	0	12	0	0
Marquis $\times$ Minturki, $F_1$ .....	12	0	0	12	0	0
Marquis $\times$ Minhardi, $F_2$ .....	978	429	105	444	55	49
Do.....	1, 029	252	85	692	33	29
Total or average Marquis $\times$ Minhardi, $F_2$ .....	2, 007	681	190	1, 136	43	39
Marquis $\times$ Minturki, $F_2$ .....	1, 138	453	152	533	53	49

#### HARDINESS IN $F_3$

Selection of individual plants was made in  $F_2$ , and the  $F_3$  lines were grown in replicated short rows from fall seeding. When sufficient seed was available, the Marquis  $\times$  Minhardi  $F_3$  lines were also grown in individual rows from spring seeding. The parental varieties, Marquis, Minturki, and Minhardi, were grown throughout the fall-sown nursery at intervals of about every eleventh row and also were included in the spring-sown nursery. As has been stated, a selection of plants in  $F_2$  was made in the early spring on the basis of strength of plant and at maturity on the basis of period of ripening. The data on winter hardiness of the various types of selections are given in Table 2.

TABLE 2.—The relation of vigor and time of maturity in  $F_2$  to hardiness in  $F_3$  in hybrids from crosses of Marquis with Minhardi and Minturki wheats at University Farm, St. Paul, Minn.

Variety or cross	Selection class in $F_2$	Number of lines	Number hardy as winter parent	Average hardiness index
Marquis.....		13		0
Minhardi.....		33		70
Minturki.....		15		67
Marquis $\times$ Minhardi, $F_3$ .....	Strong.....	45	11	28
Do.....	Weak.....	45	0	11
Do.....	Early, good seed.....	35	2	27
Do.....	Early.....	20	2	17
Do.....	Midseason.....	20	2	34
Do.....	Late.....	20	0	
Marquis $\times$ Minturki, $F_3$ .....	Early.....	20	0	
Do.....	Early, good seed.....	33	0	
Do.....	Midseason.....	20	0	
Do.....	Late.....	20	0	

The winter of 1924-25 was more severe than that of the preceding year. Marquis was completely killed as usual, while Minhardi and Minturki had an average hardiness index of 70 and 67, respectively. Forty-five  $F_3$  lines of Marquis  $\times$  Minhardi, grown from seed of strong  $F_2$  plants, had an average hardiness index of 28. Eleven of these were as hardy as the winter parent. Forty-five  $F_3$  lines grown from seed of weak  $F_2$  plants had an average hardiness index of 11, and none of these was as hardy as the winter parent. The selection of strong  $F_2$  plants in the spring and the use of the progeny from these appeared to be worth while in this case. The selection of strong and weak plants was made in an  $F_2$  row of Marquis  $\times$  Minhardi in which winter-killing was especially severe. The selections for date of heading were made in plots of the  $F_2$  of Marquis  $\times$  Minhardi and Marquis  $\times$  Minturki which were comparable, although winterkilling was less severe than in the plot where selections were made for vigor of plant. In the Marquis-Minhardi cross 55 plants which were selected for early maturity in the  $F_2$  had an average hardiness index of 23.4 in the  $F_3$ ; 20 plants selected for midseason maturity produced progeny with an average hardiness index of 34; and 20 plants selected for late maturity produced progeny with an average hardiness index of 20. The average hardiness index for all three groups was 24.8. Four lines from the early-maturing plants and two from those maturing at midseason were as hardy as the winter parent.

In the Marquis-Minturki cross, 53 plants were selected for early maturity in the  $F_2$ . The progeny of these 53 early plants had an average hardiness index in the  $F_3$  of 10.7; 20 plants selected for mid-season maturity produced progeny with an average hardiness index of 16; and 20 plants selected for late maturity produced progeny with an average hardiness index of 8. The average hardiness index for all three groups was 11.3. None of these lines was as hardy as the winter parent.

These results indicate that those plants selected for an intermediate heading period produced progeny with a higher hardiness index, on the average, than the progeny of parent plants selected for early or late heading.

The range of variability for the hardiness-index values in the  $F_3$  can be appreciated by a comparison of the results obtained from the individual hybrid lines in relation to the parents (Table 3). There were 185 lines of crosses of Marquis  $\times$  Minhardi and 93 from crosses of Marquis  $\times$  Minturki. Thirty of the 185 lines of the cross of Marquis  $\times$  Minhardi were within the range of variability for hardiness of the Minhardi parent, while 6 of the 93  $F_3$  lines of Marquis  $\times$  Minturki were within the range of variability of Minturki. It is desirable to state again that these results are averages for four replicated rows and are therefore fairly reliable.

TABLE 3.—*Frequency distribution of parent checks and of 278  $F_3$  lines from crosses of Marquis with Minhardi and Minturki wheats at University Farm, St. Paul, Minn.*

Variety or cross	Class centers for hardness index																		Number of lines	Average hardness index	
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85			90
Marquis	13																			13	0
Minhardi																				33	70.3±1.4
Minturki																				15	67.3±2.0
Marquis × Minhardi	26	23	23	20	19	18	14	3	4	5	9	7	7	4	1		1	1		185	22.4±1.0
Marquis × Minturki	29	21	13	11	5	4	3	1	1	2			3							93	11.1±1.0

Another method of comparison was used which helps to bring out the nature of the results. As the parents were grown in about every eleventh row it was possible to compare the  $F_3$  lines directly with the nearby parents. If an  $F_3$  line was as hardy as one of the nearby parent lines, it was considered practically as hardy as the parent. On this basis, 17 of the 185  $F_3$  lines of Marquis  $\times$  Minhardi were as winter hardy as the parents, but none of the 93  $F_3$  lines of Marquis  $\times$  Minturki were so winter hardy.

#### HARDINESS AND GROWTH HABIT IN $F_3$

The relationship between hardiness and habit of growth was studied for 105 Marquis-Minhardi  $F_3$  lines, where sufficient seed was available, so that they were grown from both fall and spring sowings. The distribution of the  $F_3$  lines of different growth habits in the hardiness-index classes is shown in Table 4. Five classes were used in distributing the  $F_3$  lines for growth habit when spring sowing was practiced. These are as follows:

1. Homozygous winter, where all plants failed to head when sown in the spring.
2. Heterozygous winter, where segregation occurred with a predominance of the winter type and late heading.
3. Heterozygous intermediate, where segregation occurred with rather even distribution over all periods of heading.
4. Heterozygous spring, where segregation occurred with a predominance of the spring type.
5. Homozygous spring, where all plants headed as spring wheats.

TABLE 4.—Correlation of growth habit and winter hardiness of 105  $F_3$  lines from crosses between Marquis and Minhardi wheats at University Farm, St. Paul, Minn.

Growth habit	Class centers for hardiness index																			Number of lines	Average hardiness index	
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90			
Marquis.....	13																			13	0	
Minhardi.....												4	2	2	6	4	3	6	5	1	33	70.3±1.4
Homozygous, winter.....		2				1	2	1	1	2	1	5	3	6	2				1		27	46.5±2.3
Heterozygous, winter.....						3	2	1				2	1								9	31.7±2.8
Heterozygous, intermediate.....			8	9	7	7	5	3													39	15.1±0.8
Heterozygous, spring.....		3	6	4	1	2	1	2													19	11.1±1.4
Homozygous, spring.....		6	3		2																11	4.1±1.4

This study shows that a correlation does exist between ability to survive the winter and winter habit of growth. Those lines which are homozygous for the winter growth habit have an average hardiness index of  $46.5 \pm 2.3$ . Those homozygous for spring habit of growth have an average hardiness index of  $4.1 \pm 1.4$ . The  $F_3$  heterozygous lines for growth habit were divided into three groups. Those with a predominance of winter types had an average hardiness index of  $31.7 \pm 2.8$ ; those which were like the  $F_2$  distribution had an average hardiness index of  $15.1 \pm 0.8$ ; and those in which spring types predominated had an average hardiness index of  $11.1 \pm 1.4$ .

While these results do show a general relationship between resistance to winterkilling and winter habit of growth, it is evident that some forms with a low winter resistance have the winter-growth habit, as some lines which were homozygous for winter-growth habit

killed out nearly as completely as the Marquis parent, when fall sown. Naturally, such strains are lost, because of their failure to head when spring sown, and their failure to survive when fall sown. Two of the lines when sown in the spring headed normally as late spring wheat and when sown in the fall had a fairly high hardiness index.

#### HARDINESS IN $F_4$

Selection was made in  $F_3$  on the basis of hardiness index and seed quality. As a rule, unless the seed type was particularly promising, only a single plant selection was made in each of the  $F_3$  lines which were low in winter hardiness. In those lines, however, which were as winter hardy as the winter parent in  $F_3$ , or approached this condition, larger numbers of plants were selected. Each  $F_4$  line was the progeny of a plant selected in  $F_3$ , and, as in previous generations, was grown in the fall-sown nursery in four replicated rows, each containing 25 seeds. Nearly all  $F_4$  lines were grown also as spring-sown wheats.

The frequency distribution for hardiness of the parents and crosses is given in Table 5. The severity of the winter was not greater than in 1924-25 when the  $F_3$  was grown. The hardiness index of  $F_4$  was much greater than that of  $F_3$ . This is without doubt a result of selection in  $F_3$ .

TABLE 5.—Frequency distribution for winter hardiness of parent checks and of 533  $F_4$  lines from crosses of Marquis with Minhardi and Minturki wheats at University Farm, St. Paul, Minn.

Variety or cross	Class centers for hardness index																			Number of lines	Average hardness index
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90		
Marquis	53																			53	0
Minhardi										1	4		7	9	6	6	2	2		37	66.2±1.1
Minturki													1	4	1	3	4	2	1	16	74.7±1.4
Marquis×Minhardi, F <sub>4</sub>		4	2	9	10	21	24	51	53	48	51	38	24	22	5	8	1			371	43.7±0.5
Marquis×Minturki, F <sub>4</sub>	1	2	3	7	6	11	14	27	24	12	13	16	10	10	2	2	1	1		162	41.0±0.8

To determine the extent to which hardiness in  $F_3$  is correlated with that in  $F_4$ , the hardiness index of the  $F_3$  lines from which individual plants were selected was correlated with the hardiness index of progenies of these lines in  $F_4$ . The results are presented in Table 6. The correlation coefficient was  $+0.1917 \pm 0.0282$ . While this is mathematically significant in the light of its probable error, it is very low. This low correlation is in agreement with those obtained by both Martin<sup>8</sup> and Vassar.<sup>9</sup> A high correlation could not be expected for the reason that hardiness is a complex character and without doubt is dependent upon several genetic factors. In the heterozygous lines, plants with low cold resistance will be eliminated, and the occasional plant which survives in such heterozygous lines may be one which contains genetic factors for hardiness which are much above the average of the line.

<sup>8</sup> MARTIN, J. H. Op. cit.

<sup>9</sup> VASSAR, L. P. Op. cit.

TABLE 6.—*Correlation of winter hardiness in 1925 and 1926 in crosses of Marquis with Minhardi and Minturki wheats at University Farm, St. Paul, Minn.*

1925 winter hardiness	1926 winter hardiness																			Total
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	
0.....	1			4	1	1	4	1	2		1	1	1	2						20
5.....		3		1	1	4	4	6	5	4	5	4	1	4	1					44
10.....		1	1	2	3	2	5	1	4	3	6	4	2	2		1				37
15.....		2	1	2	2	5	1	4	6	4	5	5	4	4	2					47
20.....				1	1	5	4	6	6	4	4	5	2	2	1					41
25.....				1	1	1	5	6	3	4	5	2	4	1		2		1		36
30.....			1	2	4	8	3	9	12	5	5	5	3							57
35.....						1	1	4	3			2								11
41.....			1		1			6	5	8	2	1	1	1	2					28
47.....						1	1	4	2	2		3	1	2						16
50.....						1	1	9	8	6	6		2	3		1				37
53.....				1			3	7	6	6	8	6	2	1		1				41
60.....			1		2	1	4	11	8	4	11	12	7	8		1	1			71
65.....				2		2	2	2	5	7	5	3	2			1				31
70.....								1	1	1			1							4
75.....																				0
80.....																				0
85.....									1	2		1	1	2		2				9
91.....																				0
Total..	1	6	5	16	16	32	38	77	77	60	63	54	34	32	7	9	2	1		530

$$r=0.1917 \pm 0.0282$$

As previously stated, nearly all  $F_4$  lines which were grown as winter wheats were sown also in the spring. This was for the purpose of correlating the habit of growth with the winter hardiness. The  $F_4$  lines were placed in five classes for growth habit, as in  $F_3$ . The classes were as follows: Those homozygous for winter habit, in which no plants headed when spring sown; those homozygous for spring habit, in which all plants headed when sowing was made in the spring; and three classes of heterozygotes, in which some plants headed and others failed to head. These three classes were made on the basis of the relative numbers of spring and winter types. It will be recalled that in  $F_3$  there was a close relation between winter hardiness and habit of growth. No such close relation is evident in  $F_4$ , although a slight tendency for such a relation is apparent (Table 7).

TABLE 7.—*Correlation of growth habit and winter hardiness in  $F_4$  lines from crosses of Marquis with Minhardi and Minturki wheats at University Farm, St. Paul, Minn.*

Variety or cross and growth habit	Classes for hardiness index																			Number of lines	Average hardiness index
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90		
Marquis, spring.....	53																			53	0
Minhardi, winter.....										1	4		7	9	6	6	2	2		37	66.2±1.1
Minturki, winter.....													1	4	1	3	4	2	1	16	74.7±1.4
Marquis × Minhardi, $F_4$ :																					
Homozygous, winter.....		1	1	4	4	13	19	47	46	43	42	31	19	19	3	8	1			301	45.0±0.5
Heterozygous, winter.....			1	3	3				1	1	2	3	1	1						16	36.9±2.6
Heterozygous, intermediate.....		1	1	1	1	2	2	1	1	4	3	1		1						20	39.3±2.6
Heterozygous, spring.....		2	2	2	2	1	1	2	1	2	9	2		1						22	36.6±2.7
Homozygous, spring.....			1		2	1		1	1		1	1								9	40.0±3.7
Total.....		4	2	9	10	21	24	51	51	48	51	38	24	21	5	8	1			368	43.7±0.5
Marquis × Minturki, $F_4$ :																					
Homozygous, winter.....		1	3	6	6	9	12	22	23	11	11	13	10	8	2	2	1	1		141	41.5±0.9
Heterozygous, winter.....			1			2	1	2	1		2	2		1						12	39.2±3.1
Heterozygous, intermediate.....				1		1	2													4	28.8±2.7
Heterozygous, spring.....																				0	
Homozygous, spring.....		1						1		1										3	26.7±7.5
Total.....		1	2	3	7	6	11	14	27	24	12	13	15	10	9	2	2	1	1	160	40.8±0.8

These data prove that correlation between growth habit and cold resistance is not complete. Apparently it is possible to obtain spring wheats which are as winter hardy as the most winter-resistant varieties. The heading period and winter-hardiness index of several of the hardest of these types, which were homozygous for heading when spring sown, are given in Table 8. The heading periods contain all plants which headed during certain intervals of seven days. Nearly all of the Marquis plants headed in the first week. The hybrids headed considerably later. Marquis  $\times$  Minhardi  $F_4$  line 129-1 was spring sown in both  $F_3$  and  $F_4$ . More than 100 plants were grown in  $F_3$ , and all headed during the second to the fourth week. There seems to be no doubt that this line is homozygous for ability to head when spring sown.

TABLE 8.—*Period of heading of individual plants of  $F_4$  wheat hybrids of very winter-hardy lines in which all plants headed when sown in the spring at University Farm, St. Paul, Minn.*

Parent or cross	Hardi- ness index	Weekly heading periods			
		1	2	3	4
Marquis.....	0	56			
Marquis $\times$ Minturki, 264-3.....	47		18	1	
Marquis $\times$ Minhardi, 854-1.....	57	1	5	3	4
Marquis $\times$ Minhardi, 855-1.....	42	1	14		6
Marquis.....		147		4	
Marquis $\times$ Minhardi, 129-1.....	65		13	1	2
Marquis $\times$ Minhardi, 977-1.....	47		15	5	

## DISCUSSION

A study of the interrelation of habit of growth and winter hardiness in crosses between spring and winter wheats has been made. In these studies winter hardiness is without doubt a result of cold resistance. The winter habit of growth was differentiated from the spring habit on the basis of ability to head when spring sowing was practiced. There was a close correlation between growth habit and cold resistance, although the linkage relation was not absolute. Some lines were obtained from these crosses which were homozygous for ability to head when spring sown and were also highly winter hardy as determined from actual test. These lines are similar to those of certain German investigators who have observed types which are winter hardy and also produce heads when sown in the spring. These lines probably originated from crosses of spring and winter wheats as a result of the recombination of genetic factors for cold resistance and for habit of growth.

From these results it appears that cold resistance is inherited in the same manner as other quantitative characters. A determination of the number or nature of factors involved in an expression of growth habit and cold resistance is not possible from these studies, as approximately 50 per cent of the plants were winterkilled in  $F_2$ , and it is impossible to say whether these types which were eliminated were of winter or spring habit. Without doubt the major part were of spring habit, as is shown by the close correlation in  $F_3$  between spring habit and low winter resistance.

In the  $F_3$  generation, 188 lines, each the progeny of an  $F_2$  plant from the Marquis  $\times$  Minhardi cross, were fall sown. In cases where all plants were not killed, at least one line from each  $F_3$  was grown also in  $F_4$ . Four lines bred true in  $F_3$  and  $F_4$  for a hardiness index equal to that of the winter-hardy parent. This indicates that in these crosses several genetic factors are necessary to explain the inheritance of cold resistance.

#### SUMMARY

The inheritance of growth habit and cold resistance has been studied in generations of  $F_1$  to  $F_4$  crosses of Marquis with Minturki and Minhardi wheats. Winter wheats, when spring sown, failed to head, as a rule, at University Farm, St. Paul, Minn., while spring wheats such as Marquis were entirely winter killed, as a rule, when fall sown. The winter wheats Minturki and Minhardi are two of the most winter-hardy varieties for those sections where cold is a limiting factor.

The  $F_1$  crosses headed as early as Marquis when sown in the spring and were entirely winterkilled when sown in the fall. Apparently spring-growth habit and low cold resistance are dominant over winter-growth habit and high cold resistance.

The  $F_2$  generations were sown in the fall, each cross being grown in two replicated plots. The hardiness index values ranged from 29 to 49 for the four plots, and hardiness index values 0 and 99 were obtained, respectively, for Marquis and Minhardi, where 100 represented perfect hardiness. Selection was made in the spring for vigor of plant in the Marquis  $\times$  Minhardi cross. Those plants that came through the winter without injury were called "strong" and those alive but seriously injured were designated "weak." Selection was also made at maturity, three classes being selected, early, midseason, and late.

$F_3$  and  $F_4$  hybrids were each fall sown in four replicated single rows each containing 25 seeds. An average hardiness index on the basis of a perfect stand of 25 plants was computed by multiplying each strong plant by four, each weak plant by two, and each dead plant by zero, and placing the results on a 25-plant basis. When seed was available, the  $F_3$  lines of Marquis  $\times$  Minhardi were sown also in the spring. Rows of the parents were sown about every eleventh row throughout both nurseries.

The progeny of strong  $F_2$  plants was, on the average, superior in winter hardiness to the progeny from weak ones. In both crosses the progenies of plants selected for midseason maturity were more winter hardy, on an average, than the progeny of those selected for either early or late maturity.

In  $F_3$  there was a close correlation between habit of growth and winter hardiness in the lines of Marquis  $\times$  Minhardi which were sown both in the fall and in the spring. Some lines which were homozygous for winter habit were winterkilled almost completely, and some lines, homozygous for heading when spring sown, were rather winter hardy.

Nearly all  $F_4$  lines were sown both in the fall and in the spring. In the  $F_4$  lines the differences in winter hardiness between the lines homozygous for winter or for spring habit of growth were not very great.

Cold resistance and winter habit were strongly correlated in inheritance, but the correlation was not absolute. Cold-resistant types which are capable of heading when spring sown probably are the result of a recombination of genetic factors for spring habit of growth obtained from Marquis with factors for cold resistance obtained from the winter-wheat parents, Minhardi and Minturki.

When the heading periods of lines of similar dates of sowing were compared, the hybrid lines which were homozygous for heading when spring sown, and which were highly cold resistant, matured later in all cases than Marquis.

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# THE DEFERRED SHORT-TIME TEST AS A MEASURE OF THE PERFORMANCE OF DAIRY COWS<sup>1</sup>

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## INTRODUCTION

In the official testing of dairy cows for production two types of test are commonly distinguished—the short-time test and the long-time test. The most common short-time test is for 7 days; the most common long-time test covers 12 months, or of late years, 10 months. At the present time the Holstein breeders are the only ones who make use of the short-time test. They have used it continuously and extensively under the present form of official supervision for the past 33 years (since 1894).

The long-continued and extensive use of the seven-day test may be taken as *prima facie* evidence that it possesses some merit. It has, nevertheless, some well-known faults. In the case of the more recent seven-day Holstein records the fat percentage of the milk frequently is too high to represent the usual performance of the Holstein cow. This defect of the short-time test was pointed out several years ago by Eckles (4),<sup>2</sup> who based his conclusions on experimental and observational evidence. Eckles concluded that “tests of dairy cows made for short intervals in the beginning of the lactation period can not be depended upon to indicate the normal per cent of fat produced by the cows tested.”

Another charge often made against the short-time test is that the production record does not depend upon and does not measure persistency of lactation. Practically, it is the year's performance of a cow that is of importance from the standpoint of economical milk production; obviously, two cows with equal seven-day records might and often do have widely different records for the year. The relation between the seven-day records and the year records of Holstein cows has been fully treated by Yapp (17) and by Gowen and Gowen (11).

The criticisms of the seven-day test mentioned above apply to the test as usually conducted at an early stage of lactation. It is the purpose of the present paper to examine the possibilities of the *deferred* short-time test as affording a representative record of the performance of the cow.

Certain Holstein and Guernsey records are used in this examination. Guernsey breeders have never used the seven-day test (except for six records), but the yearly records of the breed were for many years published by calendar months and they are thus usable in connection with the present problem. It is proposed to examine these records with reference particularly to the evidence which they afford as to the possibility of eliminating the objectionable features of the short-time test by deferring its application to a comparatively late stage

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<sup>2</sup> Reference is made by number (*italic*) to “Literature cited,” p. 249.

of lactation. Naturally, statistical methods have to be employed. The numerical values used from the records are those of fat percentage, milk yield, and, derived from these two, energy yield.

Energy yield is a very direct and exact measure of the work performed by the cow (5, 6, 9) and may be regarded as biologically the best measure of total production. It is estimated in terms of 4 per cent milk, designated as "fat-corrected milk" (F. C. M.), by the formula (9)  $F. C. M. = 0.4M + 15F = M(0.4 + 0.15f)$  where  $M$  is milk yield,  $F$  is fat yield, using the same unit of weight throughout, and  $f$  is fat percentage. One pound F. C. M. = 1 pound 4 per cent milk = 340 calories (2, 13, 14).

Energy yield is a measure of the *amount* of work performed and fat percentage is a measure of the *direction* in which the work is performed. For economic reasons it is sometimes advantageous to produce milk fat with a minimum production of other milk solids (milk with high fat percentage), or vice versa (milk with low fat percentage). Energy yield and fat percentage are entirely independent variables and furnish the two most essential measures of performance.<sup>3</sup>

We may consider first the relation between the short-time test and persistency, approaching the relation through theoretical or generalized considerations.

### LACTATION CURVE THEORY

By lactation curve is meant the curve describing the *rate* of milk secretion (rate of energy yield in the present connection) throughout the lactation period, or the portion of that period under consideration. The short-time test may be regarded as determining a point in the lactation curve. For example, the production for seven days may be taken as a measure of the *rate* of production per week at the middle of that week, if it is assumed that the lactation curve is linear.

The linear lactation curve may be expressed by the equation:

$$\frac{dy}{dt} = b - ct \text{-----} (1)$$

where  $y$  is yield in pounds of F. C. M.,  $t$  is time in months,  $b$  and  $c$  are constants. Curves of this type are presented in Figure 1, A. The total yield for 12 months seems to be accepted almost by common consent as a highly valuable measure of production in official test practice. Figure 1, A, is constructed to represent the 12 months' record. Obviously, from the way the figure is constructed, the area under any one of the curves between the 0 and 12 ordinates represents the year's yield for that particular curve. It is

<sup>3</sup> The biological significance of fat percentage should be held clearly in mind. It is to be regarded as an expression of the ratio ( $\times 100$ ) of the rate of fat secretion to the rate of milk secretion. Many investigators seem to have the point of view that fat yield is the result of milk yield and fat percentage. A more logical point of view seems to be that fat yield is the result of the rate of fat secretion, and that milk yield is the result of the rate of milk secretion, while fat percentage is the result of the relative values of these two rates. Fat percentage has a wider significance as a measure of performance because of its close correlation with protein percentage and energy value per unit milk.

The significance of energy yield is obvious. If we regard the cow as a machine, the *rate* of energy yield may be regarded as the *horsepower* delivered by the machine. A cow milking at the rate of 50 pounds F. C. M. per day produces the equivalent of a continuous delivery of 1.103 horsepower (1 calorie = 3,084 foot-pounds). A 1,200-pound horse at steady work may deliver about 0.9 horsepower for 10 hours per day. This is equal to a continuous delivery of 0.375 horsepower. An equivalent horsepower (i. e., 0.375 horsepower) is delivered by a cow milking 17 pounds F. C. M. per day, or 6,205 pounds F. C. M. per year.

also clear from mere inspection that a series of curves of different slopes but passing through a common point in the ordinate at  $t=6$  will each produce a figure of the same area.

If the rate of yield per month at  $t=6$  is determined, the area or year's yield is likewise determined regardless of the slope of the curve (provided, of course, it does not cut the base line). Hence, as far as persistency of lactation is concerned, there is apparently a possibility that a six-months-after-calving short-time test may possess as much merit as the year test.

That the areas under the three curves of Figure 1, A, are equal may be shown by the use of geometrical theorems—the equality of

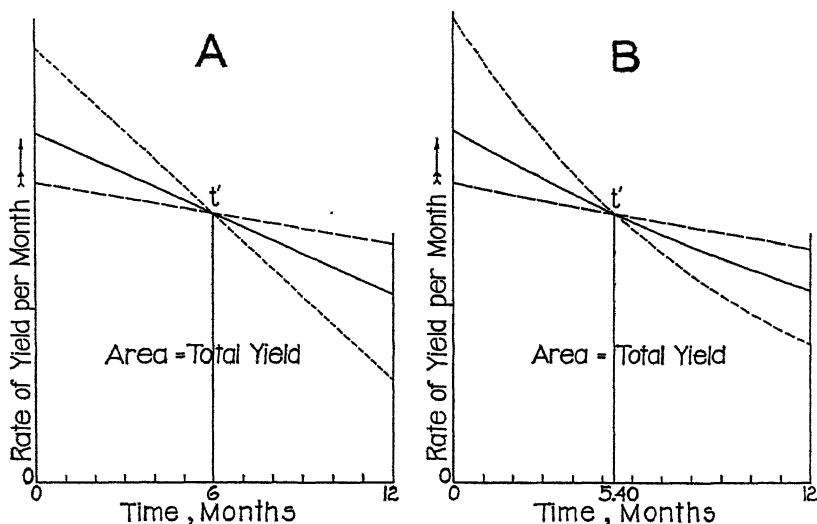


FIG. 1.—Linear and exponential lactation curves

A, linear lactation curves. The area from  $t=0$  to  $t=12$  (year's yield) is completely independent of the rate of decrease in the rate of yield (persistency) at any fixed point ( $t'$ ) in the lactation curve and in the ordinate at  $t=6$ . That is, if the rate of yield at  $t'$  is determined, the year's yield is likewise determined.

B, exponential lactation curves. The area from  $t=0$  to  $t=12$  (year's yield) is practically independent of the rate of decrease in the rate of yield (persistency) at any fixed point ( $t'$ ) in the lactation curve and in the ordinate at  $t=5.40$ . That is, if the rate of yield at  $t'$  is determined, the year's yield is approximately determined.

\* NOTE.—The ordinates  $t'$  in A and B are the same height, although the one in B appears taller. This appearance is an optical illusion associated with the slopes of the curves. The height, apparent or real, of the  $t'$  ordinate is of no concern in the relationship which the figures illustrate.

opposite angles, etc. A much more elegant and comprehensive method is by way of the differential calculus, which serves to discover the ordinate in question as well as to demonstrate the property of equal areas under the curves. Integrating equation (1) we get:  $y = bt - 0.5ct^2 + C$ . Since  $y=0$  when  $t=0$ , it is found by substitution that  $C=0$  and hence,

$$y = bt - 0.5ct^2.$$

Let  $Y=12$  months' yield,  $=12b - 72c$ .

Let  $t' =$  point in curve where  $Y$  is unaffected by variation in  $c$ .

Let  $b' =$  rate of yield at  $t'$ ,  $=b - ct'$ .

Then,

$$b = b' + ct'$$

and

$$Y = 12b' + 12ct' - 72c \dots\dots\dots (2)$$

It is necessary now to determine the value of  $t'$  at which a change in  $c$  produces no change in  $Y$ . Evidently this may be obtained by differentiating  $Y$  with respect to  $c$  in equation (2), setting the first derivative equal to zero, and solving for  $t'$ , which gives

$$\frac{dY}{dc} = 12t' - 72 = 0$$

and

$$t' = 6.$$

That is to say, a point in the curve of equation (1) at  $t=6$  fully determines the area under the curve between the ordinates 0 and 12, regardless of the value of  $c$ , or the slope of the curve. When the area is interpreted in terms of milk yield it is necessary to impose the limitation that  $b-12c$  is not negative, since a negative rate of yield does not enter into the present problem.

It appears probable that the lactation curve is better represented as somewhat curvilinear rather than linear. The exponential equation introduced in connection with the lactation curve by Brody et al. (3), satisfies the requirements in this respect very well. In Figure 1, B, are presented lactation curves of the exponential type,

$$\frac{dy}{dt} = ae^{-kt} \dots\dots\dots (3)$$

in which  $y$  and  $t$  have the same meaning as before,  $a$  and  $k$  are the limiting constants of the particular lactation curve, and  $e$  is the base of natural logarithms. In equation (3),  $k$  is the factor that is affected by persistency and it is necessary to determine the point in the curve of the equation where the year's yield is unaffected by variation in persistency, or  $k$ . This may be done by a method similar to that just followed.

Integrating equation (3) gives  $y = -\frac{a}{k} e^{-kt} + C$ . Since  $y=0$  when  $t=0$ , then  $C = \frac{a}{k}$ , and hence,

$$y = \frac{a}{k} (1 - e^{-kt}).$$

Let  $Y=12$  months' yield,  $= \frac{a}{k}(1 - e^{-12k})$ .

Let  $t'$  = point in curve where  $Y$  is unaffected by variation in  $k$ .

Let  $a'$  = rate of yield at  $t'$ ,  $= ae^{-kt'}$ .

Then

$$a = a' e^{kt'}$$

and

$$Y = \frac{a' e^{kt'}}{k} (1 - e^{-12k}) \dots \dots \dots (4)$$

Differentiating  $Y$  with respect to  $k$  in equation (4) and setting the first derivative equal to zero, gives

$$\begin{aligned} \frac{dY}{dk} &= \frac{a' e^{kt'}}{k} 12e^{-12k} + (1 - e^{-12k}) \frac{ka't' e^{kt'} - a' e^{kt'}}{k^2} \\ &= Y \left[ \frac{12e^{-12k}}{1 - e^{-12k}} + t' - \frac{1}{k} \right] = 0. \end{aligned}$$

and

$$t' = \frac{1}{k} - \frac{12e^{-12k}}{1 - e^{-12k}}.$$

The value of  $t'$  varies with  $k$ , so that there is no fixed value as in equation (1). One recourse is to take a mean value for  $k$ . For cows on official test it appears (3, 7, 10) that  $k=0.05$  approximately. When  $k=0.05$ ,  $t'=5.40$ . The indication is, therefore, that the short-time test should be deferred to about 5.4 months after the start of the year's record in order to most accurately indicate the year's record.

It is evident to anyone familiar with the course of milk secretion with advance in lactation that such a deferred short-time production is subject to many disturbing factors. This is true also of the year's yield. In the Guernsey records <sup>4</sup> the correlation between daily energy yield of the sixth full calendar month of the lactation and energy yield for the year has been computed (7) and found to be  $r=0.928 \pm 0.002$ . There is little doubt that under similar environmental conditions a seven-day test conducted during the fifth month of the year-period would prove to be fully as highly correlated with the year's record as indicated by the above coefficient.

It seems, therefore, that the objection to the short-time test (as compared with the year test) namely, that it does not depend <sup>5</sup> upon persistency of lactation, may be disposed of by deferring the test to the fifth month of the lactation.

It has been assumed thus far that the year test is the ideal. But if we may judge from the practice of the milk producer, rather than the Advanced Register breeder, it is desirable to so manage the cow that she will bear a calf about every 12 months and have a dry period of about six weeks during each calving interval. There is other evidence (8, 15) that such practice is consistent with the most economical production of milk. From Figure 2, it appears that in ordinary dairy practice a short-time test will afford the best index of production if it is conducted during the fourth month of lactation.

<sup>4</sup> All 365-day original entry records of volumes 33, 34, and No. 1 volume 35 of the Guernsey Herd Register (1) starting within 60 days after calving and in which conception did not recur within six months after calving.

<sup>5</sup> The year's record depends in part upon persistency but the year's yield of itself is a poor measure of persistency. The correlation between the year's yield and persistency [ $k$  of equation (3)] is  $r=-0.226 \pm 0.016$ . That the actual persistency value may be estimated with greater accuracy from an early seven-day test than from the bare figure of the year's yield is shown by the correlation between the initial rate of yield and persistency [ $a$  and  $k$  of equation (3)]  $r=0.535 \pm 0.012$ .

The conclusions derived from Figure 2 are based on a calving interval of 12 months. In commercial milk production practice the calving interval naturally varies; but, accepting 12 months as the best length of the interval, the fourth-month test is still justified, even though the calving interval is shorter or longer than a year. From a biological standpoint, what is needed is a measure of pro-

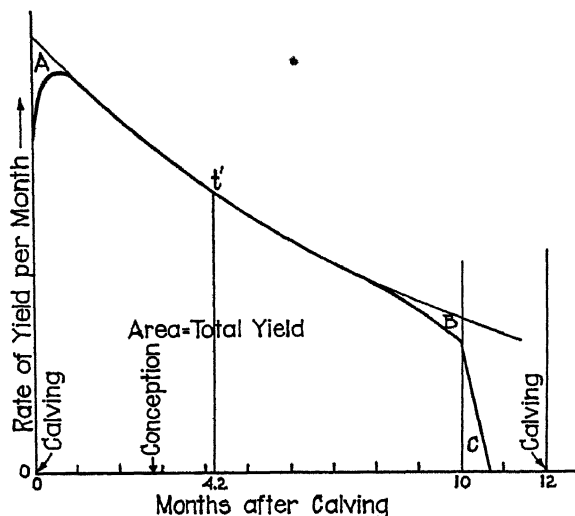


FIG. 2.—Ordinary average lactation curve (diagrammatic)

The upper (lighter line) curve is the curve of equation (3) plotted for a value of  $k=0.1$ , since it is found under common practice that the average rate of decrease in milk yield with advance in lactation is around 9 to 10 per cent per month. The lower (heavier line) curve is to represent the realized lactation curve. For the most part, it is coincident with the curve of equation (3). Immediately following calving there is a deficiency in the rate of yield required by the equation which results in the area A. This is perhaps due to a residual inhibition from the preceding pregnancy. In the late pregnancy following there is again an appreciable deficiency in the rate of yield which results in the area B. This is probably due to an inhibition associated with the current pregnancy. Finally, starting at the ordinate 10, the cow is dried up in preparation for the next lactation. A yield represented by the area C is produced during this stage.

If it is assumed that  $A+B=C$  the lactation yield will be in accordance with equation (3) from  $t=0$  to  $t=10$ . As an approximation equation (3) may be used between these time limits in determining the point ( $t'$ ) in the curve at which the area is independent of variability in  $k$ . By the method used above in the text it is found that  $t' = \frac{1}{k} - \frac{10e^{-10k}}{1-e^{-10k}}$ . For  $k=0.1$ ,  $t'=4.2$ . A short-time record at this stage of lactation should of itself afford a valuable measure of production. The ratio of the lactation yield (that is, 10 months by

the equation) to the rate of yield per month at  $t=4.2$ , is given by  $\frac{a}{k} \frac{(1-e^{-10k})}{ae^{-4.2k}}$ . Where  $k=0.1$  this ratio = 9.02.

The corresponding ratio to the rate of yield per week is 41.8. The ratio is only slightly affected by ordinary differences in  $k$ .

ductive capacity which is physiologically sound, even though it may be faulty from a cost-accounting standpoint. The cost accountant is interested in the average production per unit time over the calving interval. But the calving interval is dependent upon the recurrence of conception, which is independent of productive level (8). Reproductive capacity is a very important matter, but it is a separate problem from lactation capacity. The fourth-month test, therefore, holds its merit as a biological measure of the milk productive capacity of the cow.<sup>6</sup>

<sup>6</sup> It is quite possible that an early short-time test may be a better biological measure of lactation capacity than the deferred short-time test, or year test itself, if environmental conditions which influence the lactation curve are unfavorable or irregular. The ideal biological measure would seem to be one describing the initial value and slope of the lactation curve under standard environmental conditions.

As far as energy yield is concerned it seems from the theoretical and practical solutions given, that the deferred short-time test has much merit. Let us now examine the case with respect to fat percentage. Evidence here is available from both the Guernsey and Holstein published advanced registry records.

#### FAT PERCENTAGE OF GUERNSEY RECORDS

Both fat and milk yields of farrow Guernsey cows follow the curve of equation (3), although not as closely as does energy yield (10). The stage of the record at which the fat percentage at the time is the same as the fat percentage for the year may be computed theoretically from the fat and milk lactation curves, as follows. Let  $a_1e^{-k_1t}$  represent the rate of fat yield and  $a_2e^{-k_2t}$  the rate of milk yield. Then the time ( $t$ ) at which the fat percentage of a short-time test should be the same as the fat percentage for the year test is given by the value of  $t$  in the equation,

$$\frac{100a_1e^{-k_1t}}{a_2e^{-k_2t}} = \frac{100a_1 \frac{1 - e^{-12k_1}}{k_1}}{a_2 \frac{1 - e^{-12k_2}}{k_2}}$$

OR

$$e^{(k_2 - k_1)t} = \frac{k_2(1 - e^{-12k_1})}{k_1(1 - e^{-12k_2})}$$

If the group values found (10) for  $k_1$  and  $k_2$ , namely,  $k_1 = 0.034202$  and  $k_2 = 0.052347$ , are taken, it is found that  $t = 5.48$ . This value is practically identical with the point of closest correlation between the short-time yield and the year's yield. Hence, it appears that as far as the customary year record is concerned a deferred short-time test conducted during the fifth month should be directly representative of the fat percentage.

On the basis of the actual records (above noted) the relation between the several monthly fat percentages and the yearly fat percentage may be examined. The records are considered in two groups according as the test was conducted under the one-day or two-day monthly supervision plan. Records from tests conducted partly under one and partly under the other plan are not used. The statistical constants derived from the records are given in Table 1, and these constants are graphically shown in Figure 3.

It may be noted that the mean fat percentage curve of Figure 3 shows a tendency to a convex upward form, whereas the fat percentage curve derived from the exponential lactation curves for fat and milk as given in Figure 1, B, is very slightly convex downward. This discrepancy is probably associated with the season of calving (16, *fig. 3*).

The chief item of interest is the correlation between the one-day or two-day fat percentage and the yearly fat percentage. The change in the coefficient of correlation with advancing lactation is fairly regular in the case of the two-day test (*fig. 3*), and if the curve is smoothed it is found that the highest correlation is reached at about five months after calving. The correlation indicated at this time is about 0.83. This indicates that a five-months' deferred test should give a fairly accurate value of the year's fat percentage.

## FAT PERCENTAGE OF HOLSTEIN RECORDS

As previously stated, it is known that many seven-day records are misrepresentative of the *usual* performance of the cow, particularly with respect to the fat percentage. If the seven-day records from tests conducted at successively later stages of lactation are considered, it may be possible to find a stage where the records seem to be more representative. The question of a standard by which to judge the representativeness of the record arises. One possibility is to take as a standard the seven-day records made early in the history of advanced registry testing, before the practice of specially fitting the cow for the seven-day test had come into operation.

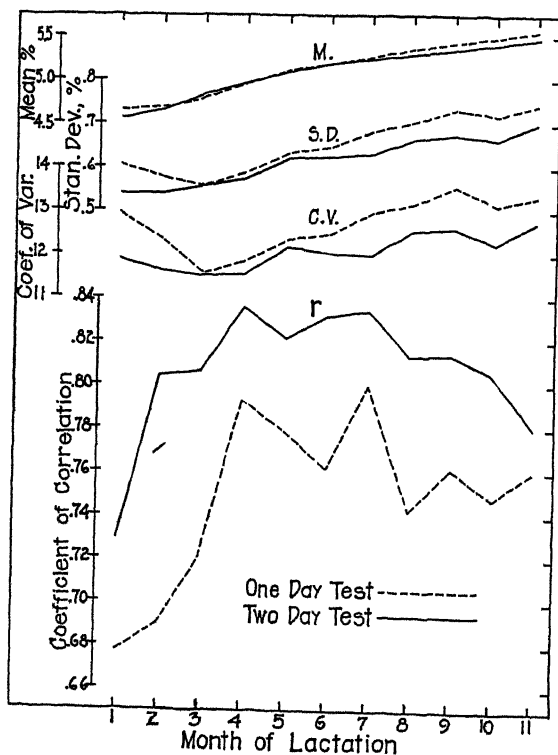


FIG. 3.—Constants of monthly fat percentages and correlation between monthly and yearly fat percentages—Guernsey records

The fat percentage and milk yield constants of certain early records (1894–1898) are given in Table 2. Table 2 gives also similar constants for certain later seven-day records (1919–1921) classified according to the stage of lactation. The mean fat percentage of the early records is 3.280 while that of the recent records is 3.485, including records started 6 to 99 days after calving. Records started at 6 to 9 days after calving have a mean of 3.756. The mean fat percentage continues to decrease up to 60 days after calving. Taking the early records as an index of normalcy, the fat percentage at this stage (60 days after calving) is quite normal. There is naturally some increase in fat percentage with advance in lactation (16).

TABLE 1.—Statistical constants of monthly and yearly fat percentages for Guernsey records<sup>a</sup>

Period of lactation	Mean		Standard deviation		Coefficient of variability <sup>b</sup>		Coefficient of correlation between monthly and yearly fat percentage	
	One-day test	Two-day test	One-day test	Two-day test	One-day test	Two-day test	One-day test	Two-day test
First month.....	Per cent 4.660±0.025	Per cent 4.550±0.012	Per cent 0.003±0.018	Per cent 0.539±0.008	12.95±0.38	11.84±0.18	0.677±0.022	0.728±0.010
Second month.....	4.684±.023	4.656±.011	.579±.016	.540±.007	12.36±.34	11.59±.16	.680±.020	.804±.007
Third month.....	4.804±.022	4.841±.011	.557±.015	.557±.008	11.59±.32	11.50±.16	.722±.019	.808±.007
Fourth month.....	4.969±.023	4.963±.011	.589±.016	.574±.008	11.85±.33	11.56±.16	.792±.015	.836±.006
Fifth month.....	5.120±.025	5.097±.012	.631±.017	.621±.009	12.32±.34	12.18±.17	.778±.015	.821±.006
Sixth month.....	5.190±.025	5.191±.012	.646±.018	.624±.009	12.45±.34	12.02±.16	.761±.016	.831±.006
Seventh month.....	5.277±.027	5.256±.012	.684±.019	.631±.009	12.90±.36	12.00±.17	.800±.014	.884±.006
Eighth month.....	5.360±.028	5.299±.013	.707±.019	.667±.009	13.19±.36	12.59±.17	.743±.017	.813±.007
Ninth month.....	5.416±.029	5.359±.013	.736±.020	.676±.009	13.58±.37	12.61±.17	.762±.016	.814±.007
Tenth month.....	5.481±.028	5.407±.013	.720±.020	.683±.009	13.13±.36	12.25±.17	.748±.017	.805±.007
Eleventh month.....	5.574±.029	5.494±.014	.744±.020	.702±.010	13.35±.36	12.77±.17	.761±.016	.780±.008
Year.....	5.069±.019	5.047±.010	.478±.013	.490±.007	9.44±.26	9.72±.13	-----	-----

<sup>a</sup> The number of records taken for the different test periods were as follows: One-day test, first month, 267; subsequent months and year, 301; two-day test, first month, 1,000; subsequent months and year, 1,205.

<sup>b</sup> The probable errors of the coefficients of variability in Tables 1 and 2 are computed by the approximate formula  $E_{cv} = 100 E_v / M$ .

TABLE 2.—Seven-day Holstein-Friesian records classified according to time after calving at which started

Time after calving	Chart (fig. 4)	Number of records <sup>a</sup>	Fat percentage			Fat percent- age <sup>b</sup>	Milk yield			Mean F. C. M.
			Mean	Standard deviation	Coefficient of variability		Mean	Standard deviation	Coefficient of variability	
6 to 99 days.....	A	277	3.280±0.0166	0.410±0.0117	12.49±0.36	3.254	Pounds 365.0±3.71	Pounds 91.5±2.62	25.05±0.76	Pounds 324.7
6 to 9 days.....	B	12,048	3.435±0.0028	.449±.0020	12.87±.57	3.469	405.1±.53	85.5±.37	21.18±.91	372.8
10 to 19 days.....	C	2,106	3.756±0.0089	.547±.0057	14.56±.15	3.734	391.9±1.10	73.1±.82	20.08±.21	376.2
20 to 29 days.....	D	3,451	3.525±0.0050	.432±.0035	12.20±.10	3.507	412.8±.97	84.0±.69	20.49±.17	382.2
30 to 39 days.....	E	1,825	3.425±0.0060	.378±.0042	11.04±.12	3.409	417.5±1.39	88.3±.98	21.13±.23	390.5
40 to 49 days.....	F	2,132	3.366±0.0056	.386±.0040	11.47±.12	3.353	410.5±1.29	88.1±.91	21.46±.22	370.8
50 to 59 days.....	G	1,083	3.330±0.0076	.372±.0054	11.09±.16	3.343	402.2±1.83	89.1±1.29	22.15±.32	362.6
60 to 69 days.....	H	565	3.330±0.0108	.382±.0076	11.47±.23	3.319	395.2±2.45	86.2±1.73	21.81±.44	357.0
70 to 79 days.....	I	392	3.345±0.0136	.398±.0096	11.91±.29	3.330	381.1±2.77	81.3±1.96	21.33±.51	342.8
80 to 89 days.....	J	494	3.366±0.0103	.340±.0073	10.09±.22	3.352	374.0±2.89	78.8±1.69	21.08±.45	337.7
240 to 269 days.....	K	1,012	3.485±0.0093	.438±.0066	12.56±.19	3.465	329.4±1.76	83.1±1.25	25.23±.38	302.9

<sup>a</sup> The records of chart A are not classified as to time after calving. They are original entries of the first 277 cows entered under the present system of official supervision, vols. 1-9 of the Holstein-Friesian Advanced Register (12). Charts C and D are original entries of vol. 31. Charts E-J are original entries of vols. 30-32. Charts C-J are combined in Chart B. This combination is not quite proper since part of the records came from one volume and part from three volumes. Chart K is derived from the "special records" of vols. 24-31 (12).

<sup>b</sup> Weighted by milk yield.

Another way in which the records may be judged is in connection with the relation between fat percentage and milk yield. It may be assumed that energy yield should be constant at various fat percentages if conditions are normal. The milk yield curve is accordingly  $y = A/(2.66 + f)$ , where  $f$  is fat percentage and  $A$  is a constant taken as the average energy yield (in units of 51 calories) for the entire group. That is,  $A = \Sigma n M_o (2.66 + f) / \Sigma n$ , where  $M_o$  is the observed milk yield at the given fat percentage,  $f$ , and  $n$  is the frequency.

In Figure 4, chart A, this curve is given for the early age-corrected records,  $A$  having the value 2.47.

The fat percentage frequency distribution of these early records is also given in chart A. The smooth frequency curve is merely a free-hand sketch.

Similar graphic presentation for the other seven-day records of Table 2 is given in Figure 4, charts B to K. The milk yields in these charts are not corrected for age and the values of  $A$  may be had from the respective values given in the last column of Table 2, by multiplying by 6.66. The smooth frequency curve is repeated from chart A as a basis of comparison.

By studying the charts of Figure 4, one may form an idea as to when and to what extent the seven-day records conducted at various stages of lactation approach representative values. The comparison is of course a rough one. Certain yearly records<sup>7</sup> are given in chart L as of interest.

Judging by the data of Table 2 and Figure 4 it would seem that deferring the seven-day test to 60 days or more after calving would eliminate the exceptional conditions and misrepresentative features of the seven-day test as conducted a few days after calving.

### SUMMARY

Two objections have been raised to the record of the seven-day test conducted shortly after calving; (1) that the fat percentage obtained is not representative, and (2) that the record is not dependent on persistency of lactation. Data from the Guernsey and Holstein advanced registry are examined with respect to the possibility of eliminating these objections by deferring the test to a later stage of lactation. It appears that the objection on the score of the fat percentage may be overcome by deferring the test to 60 days or more after calving. The objection on the score of persistency may be overcome by deferring the test to the fifth month of lactation. To best represent the practically useful lactation capacity of the cow (in distinction to the maximum record of advanced-registry practice) the short-time test should be conducted during the fourth month of lactation.

<sup>7</sup> All long-time records, including reentries, from vols. 24-30, Holstein-Friesian Advanced Register (1912-1919) (12).

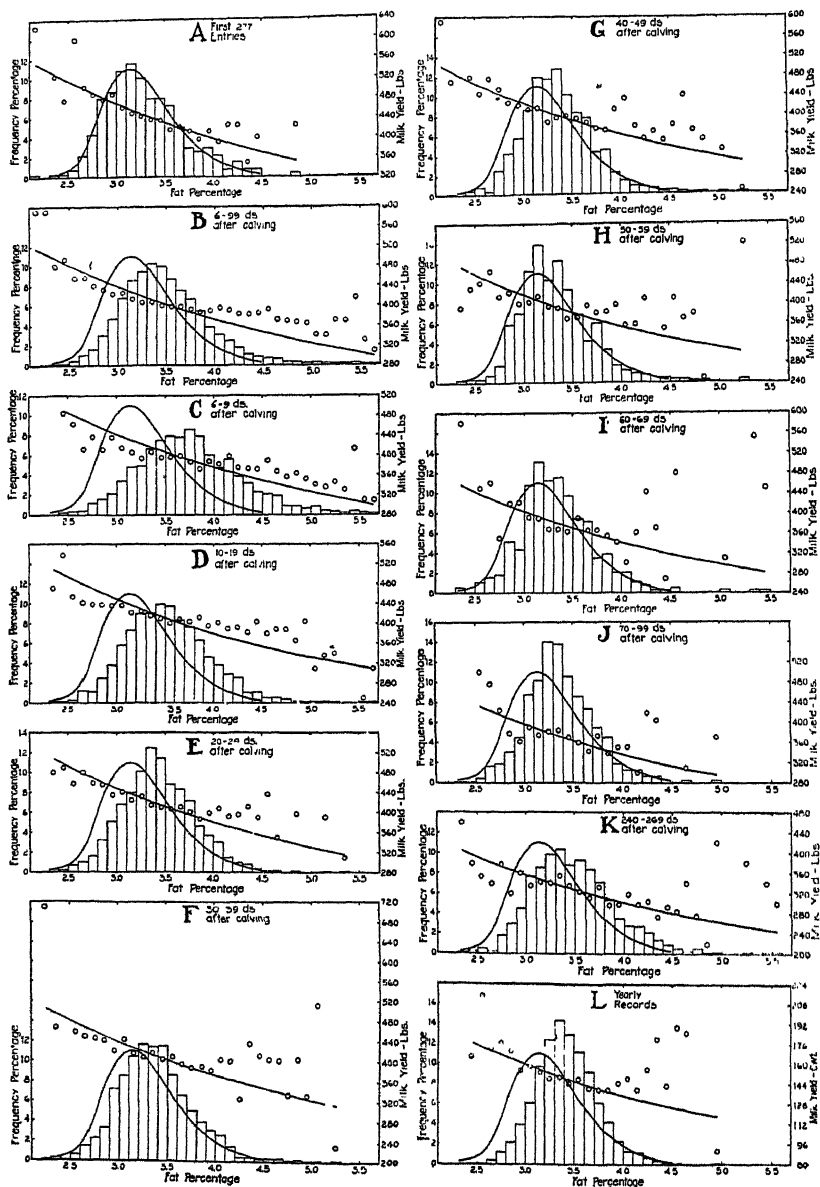


FIG. 4.—Frequencies (columns) and mean milk yields (circles) at various fat percentages for Holstein record

Chart A represents the seven-day records of the first 277 cows admitted (1894-1898) to the advanced register under the present system of official supervision. Charts B to K represent seven-day records classified according to the time after calving at which the record was started. Chart L represents yearly records. The free-hand frequency curve of chart A is repeated in charts B to L. The milk yields in chart A have been corrected for age, increasing the average yield to 122.43 per cent of the figure given in Table 2. The milk yields in charts B to L have not been corrected for age.

Observations at the following fat percentage classes are not shown in the charts indicated:

Classes.....	5.75	5.95	6.05	6.25	6.35	6.45	6.65	6.95
Charts.....	B, G	B, C	B, D	B, C	B, C	B, C	B, C	B, C

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# PROTECTION AFFORDED THE SKIN AGAINST SUNBURN BY TEXTILE FIBERS<sup>1</sup>

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## INTRODUCTION

Recent interest in the effect of ultra-violet rays on the human organism suggested a study to determine the protection from sunburn afforded the skin by textile fibers. No records could be found of any work of this nature having been done under controlled conditions.

Some experiments were performed by Hess<sup>2</sup> to determine the ability of certain fabrics, suitable for infants' clothing, to transmit ultra-violet rays. A carbon arc lamp was used as the source of radiation and rats were employed as subjects. The results were determined wholly by the biological effect of the rays on the animal organism. The application of the above research, however, to this problem of protection against sunburn is not direct.

Since work has been done on the ability of rayon, the artificial textile fiber, to transmit ultra-violet rays, it was not included in this work.

## THE PROBLEM

The object of this investigation was to determine the relative ability of the textile fibers wool, silk, linen, and cotton, to screen out ultra-violet rays from direct sunlight and from artificial light sources. It was necessary to work with the fibers in the form of fabrics.

## METHOD OF PROCEDURE

In choosing the materials with which to experiment an effort was made to obtain fabrics woven of wool, silk, linen, and cotton, of the same weight and containing the same number of threads per inch, in which case the spaces between the threads would have been the same.<sup>3</sup> This was impossible and could be done only by having the fabrics woven according to definite requirements.

The problem was arranged so as to determine the ratio of the time required to sunburn the skin through certain fabrics to the time required to produce a sunburn of equal intensity upon the unprotected skin under the same controlled conditions. This ratio was accepted as the coefficient of protection of the fabric. The difference in protection by the fabrics not due to difference in construction was accepted as the coefficient of protection of the fiber in the fabric.

The lightest weight wool fabric, plain weave, was chosen and this was matched as nearly as possible with respect to weight and number

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<sup>2</sup> HESS, A. F., and WEINSTOCK, M. A STUDY OF LIGHT WAVES IN THEIR RELATION TO RICKETS. *Jour. Amer. Med. Assoc.* 80: 689. 1923.

of picks and ends per inch, with similar fabrics of silk, linen, and cotton. The problem of the absorption due to color was avoided by choosing only white materials. Plain weave, which contained rectangular spaces between the threads and thus permitted the measurement of these threads, was used.

A complete textile analysis of fabrics used in the initial work was made. The data secured in these analyses are reported in Tables 1, 2, 3, and 4. Table 1 includes the commercial names and descriptions of fabrics; Table 2 analyses of the yarns used in their construction; and Table 3 gives detailed facts concerning the fibers used in yarns. Column three in Table 4 shows the per cent of interspace or the ratio of the total area of the fabric to the interspace included.

TABLE 1.—*Description of fabrics used in experiment*

Name of fabric	Fiber	Width in inches	Price per yard	Weave	Color	Weight (4 by 4 inch piece)	Breaking strength		Finish
							Warp	Filling	
Nun's veiling	Wool	44	\$1.95	Plain	White	Grams 1.000	Pounds 18	Pounds 9	Bleached soft.
Pongee	Wild silk	36	2.25	do	do	.586	25	21	Bleached, slightly gummed.
Handkerchief	Linen	36	1.65	do	do	.534	12	8	Bleached, soft.
Muslin	Cotton	36	.17	do	do	.816	32	16	Starched, bleached.

TABLE 2.—*Description of yarn in fabrics used in experiment*

Fabric	Yarn, size	Number per inch		Width in inches		Breaking strength		Twist per inch		Ply, warp filling
		Warp	Filling	Warp	Filling	Warp	Filling	Warp	Filling	
Wool	54	70	65	0.01040	0.00950	Ounces 8	Ounces 6	11	6	Single.
Silk	16/18	73	72	.00797	.01007	10	9	4	0	Do.
Linen	28	94	72	.00587	.00562	10	9	16	25	Do.
Cotton	40	78	72	.00521	.00625	16	9	19	13	Do.

TABLE 3.—*Length, tensile strength, stretch, and quality of fibers in yarn*

Fabric	Number of fibers in yarn		Length of fibers		Tensile strength of fibers		Stretch of		Quality of fibers
	Warp	Filling	Warp filling		Warp	Filling	Warp	Filling	
Wool	40	25	0.5 to 3 inches		Grams 11.61	Grams 5.59	Cm. 25	Cm. 13.0	Very good.
Silk	25	25	Length of piece		5.85	5.52	13	12.6	Good.
Linen	30	25	0.5 to 3 inches		6.45	6.46	15	13.0	Medium.
Cotton	34	45	0.5 to 2 inches		5.59	4.30	13	10.0	Do.

It was decided to test the effect of exposure to direct sunlight and to the lamp on the same subjects. So far as the quality of burns produced was concerned no difference could be detected between the burn produced in direct sunlight and by the ultra-violet lamp.

TABLE 4.—*Comparison of space covered by the threads with the space between the threads in fabrics studied in experiment*

Fabric	Space covered by thread (square inches)	Interspace (square inches)	Ratio of the total area of the fabric to the interspace included
Wool.....	0.89820	0.10180	9.80
Silk.....	.9192	.0827	12.08
Linen.....	.81820	.18180	5.50
Cotton.....	.81140	.18860	5.30

It seemed especially desirable to devise a physical test that could be used as a means of indicating approximately the necessary time of exposure, thereby eliminating the necessity of so many preliminary exposures. With this in view it was suggested that possibly the ability of these materials to transmit visible light which could be tested by photometry, could be used in a measure as a test of their ability to transmit ultra-violet rays, since these rays are shorter than the rays in the visible part of the spectrum. Furthermore, since the interspace between threads is usually many times greater than the wave length of ultra-violet rays, it might follow, provided the thread transmitted no light, that the ratio of the area covered by the threads to the area of the interspace would be a measure of the protection afforded.

Tests made on photographic paper of the printing-out type indicated that the paper used was sensitive only to the ultra-violet rays, and that the ratio of the time required to produce a given tone on the paper when shielded by the fabric in question to the time required to produce the same color under the unscreened light might indicate the ratio sought.

Since measurements were to be made by means of comparing the color tone of the effect of light on sensitized paper and on the skin, it was very important that the operator be able to accurately match color. Tests were made by exposing one-half inch of the printing-out paper to the light for a few seconds, one-half inch more for an additional equivalent length of time, and so on, until two whole sheets of paper had been exposed, the first strip being thereby exposed for the full time, and the time for each succeeding strip being decreased by an equal number of seconds. This gave a graduation in tone from white to very dark brown. The strips were numbered on the back and the sheets were cut in strips, 20 in all. Since the operator was able to arrange these strips in order according to tone, her ability to match color within at least a 5 per cent error was established. All light-exposed paper was fixed without toning in hypo solution at 22° C., and rinsed in running water.

#### EXPERIMENTAL WORK

A number of persons were tested to determine their sensitiveness to sunburn, both in direct sunlight and under the lamp. The arm of the subject was covered with a brown woolen sleeve from which a small piece about 1-inch square had been cut. Tests were made with a 5-minute exposure to direct sunshine, during the month of

June, with no perceptible results. The time of exposure was increased until a good burn was obtained. In all the work done the time of exposure was regulated so that blistering the skin was prevented and the burns made were of intensity sufficient to result only in a deep reddening, or erythema, which was followed by peeling and a subsequent tanning. Four of the subjects tested burned with a 30-minute exposure; one required a 45-minute exposure to produce a burn of the same intensity; and one subject was found who showed no evidence of burning even after an exposure of one hour. This person was of medium-light complexion and perspired freely. Contrary to the opinion commonly held, it was not found that the fairest skin always burned in the shortest time.

It was found that approximately only one-tenth as much time was required to obtain a burn under the quartz mercury lamp with the skin 1 foot from the lamp and a voltage over the terminals of 70 to 80, as was required to produce a burn of the same intensity in direct sunlight, between the hours of 11 and 3 on a bright day, during the months of June, July, and August. An air-cooled quartz mercury arc lamp was used, and tests were made only after voltage became constant.

The screening effect of the fabrics to visible light was obtained by means of the photometer; and the intensity of the light transmitted by the fabric was checked with the candlepower of the unscreened light. The fabric was interposed directly in front of one of the openings in the photometer screen, being fastened to it by means of elastic bands. In this manner the following ratios were obtained:

Fabric	Protective ratio
Wool.....	7.07 to 1
Silk.....	7.11 to 1
Linen.....	4.39 to 1
Cotton.....	5.86 to 1

These figures show that the wool and silk fabric transmitted only one-seventh, the linen a little less than one-fourth, and the cotton more than one-fifth of the incident visible light.

The percentage of interspace in the fabric or the ratio of the space between the threads to the space covered by the threads was determined by means of a micrometer microscope. By this method only an approximate determination could be made. Ends of fibers protruding into the interspace tend to cut down the space, but the amount of this reduction could not be measured; untwisted yarns permit passage of light through them; and the uneven line produced by the twisted thread influences the area of the interspaces. Assuming that the light passed through the interspaces and only through the interspaces, the ratios of protection offered by these fabrics would be:

Fabric	Protective ratio
Wool.....	9.8 to 1
Silk.....	12.0 to 1
Linen.....	5.5 to 1
Cotton.....	5.3 to 1

From the above it is plain that about one-fifth of the light might pass through the interspace in the case of the cotton and linen fabrics, whereas with wool and silk the quantity was somewhat less. These tests show that the number of picks and ends per inch can not be taken as a measure of closeness of weave unless the size of the  
(See Table 2.)

As previously stated, the screening effect of these fabrics to ultra-violet light was determined by means of a printing-out paper. The color obtained by 15 seconds' exposure of the paper to the rays of the lamp was decided upon as the best color to match. With a 15-second period as a basis therefore, the fabrics were tested to determine the ratio of the time required to obtain the same color tone when the paper was screened with the fabric in question. Exposures were made 1 foot from the lamp with a relatively constant voltage of 70 to 80.

For this work it was found necessary to keep either the paper or the fabric moving slightly in order to secure a uniform color tone.

The first four fabrics tested as shown in Table 5 gave the following results:

Fabric	Protective ratio
Wool.....	8.5 to 1
Silk.....	13.0 to 1
Linen.....	3.0 to 1
Cotton.....	4.0 to 1

That is, it took 8.5 times as long, or 2 minutes and 7 seconds, to obtain the same effect on the paper used when screened by the wool fabric as was obtained in 15 seconds' exposure of the unprotected paper.

TABLE 5.—*Coefficient of protection of textile fabrics*

Fabric	Coefficient determined by—			
	Photometer	Measurement of interspace	Action of ultra-violet rays on sensitized paper	Action of sunlight on skin
Wool.....	7.07	9.80	8.50	20.00
Silk.....	7.11	12.00	13.00	20.00
Linen.....	4.39	5.50	3.00	3.00
Cotton.....	5.86	5.30	4.00	4.00

The above ratios were used as a basis for time of exposure of the skin to direct sunlight when protected by the different fabrics. By using the ratio obtained by the action of ultra-violet light on sensitized paper good burns of an intensity equal to the control were secured through cotton and linen.

Several unsuccessful attempts were made to secure burns through the silk and wool fabrics in direct sunlight. No burns were obtained of sufficient intensity to show discoloration for more than 48 hours, although the ratio of 6 to 1 used required 4 hours and 15 minutes exposure. A 45-minute exposure was necessary to secure a positive burn on the unprotected skin of the subject.

An unscreened control of correct time for the individual subject was run parallel with every fabric test. This permitted the direct comparison of results and eliminated errors which might be due to uncontrollable conditions, such as the quantity of ultra-violet rays in the source of light at the particular time of exposure, and the individual's resistance to the effect of these actinic rays. The subject upon whom the largest number of the tests was made varied in her sensitiveness to sunburn. Twice during the 18 months through

which the experiments were continued, this subject showed no signs of sunburn under double the normal time of exposure, and the same controlled conditions of distance and voltage.

The fabrics were next tested under the lamp, on the same subjects, as illustrated in Figure 1. It was found that the ratios used in direct sunlight tests gave the same results under the lamp with the time of exposure reduced to one-tenth. Thus it required an exposure of  $1\frac{1}{2}$  hours to direct sunlight to secure the same intensity of burn through

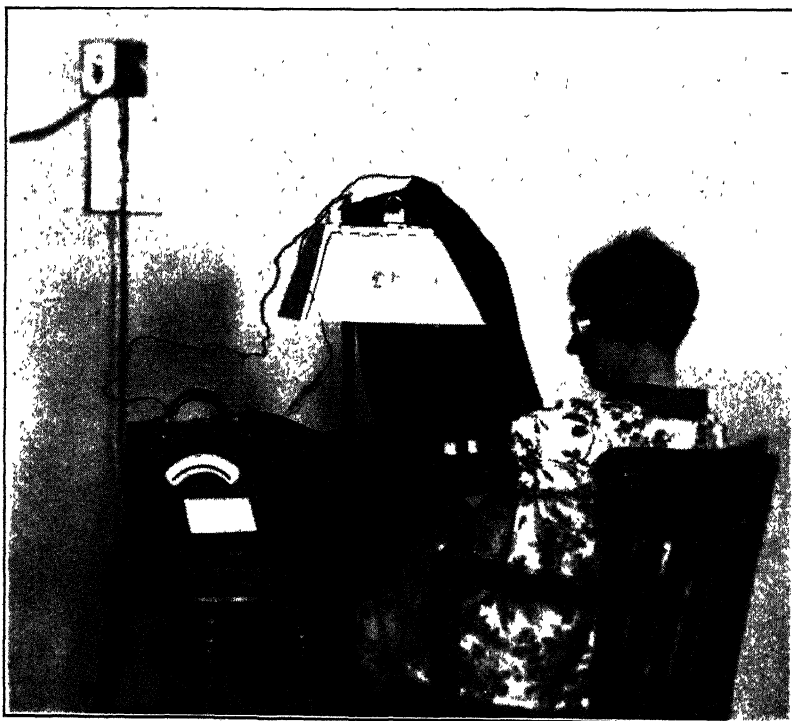


FIG. 1.—Method used in testing fabrics under the lamp

linen as was obtained in 9 minutes under the lamp. A burn that required 2 hours' exposure in direct sunlight was produced in 12 minutes by the rays of the lamp.

In tests of the silk fabric the ratio was increased to 13 to 1, the same ratio obtained by screening printing-out paper from the rays of the lamp, without perceivable results. It was found that a ratio of 20 to 1 was required to obtain a burn through this fabric.

The same method was used in testing the wool fabric, as illustrated in Figure 2, until by experiment it was found that it also required a ratio of 20 to 1.

#### SCREENING TESTS

Screening tests were made of these fabrics by the use of the spectrograph and the quartz mercury arc lamp. The fabric was fastened to a screen and interposed directly in front of the collimator, the prism having been set for minimum deviation. Under 5 seconds' exposure

for control and the ratios obtained by means of the lamp and the printing-out paper for time of exposure through the different fabrics, the ratio of 3 to 1 for linen was found to be sufficient to give a spectrograph (through the fabric) of as great a density as the unscreened control. The ratio of 4 to 1 was only slightly low for cotton. These ratios had proved correct for ratio of time of exposure to produce a



FIG. 2.—Two burns obtained with lamp; control (above), and through wool fabric (below). Ratio 20 to 1

burn on the skin both in direct sunlight and under the quartz mercury arc lamp.

The spectrographs of these four fabrics showed that none of the fabrics absorbed any of the rays of the spectrum, the range of which was between the limits of 500 and 250 microns. The fabric caused a scattering of the rays through defraction, which was very decided in cases of long-time exposure. Tests of a printing-out paper made by means of the spectrograph proved that it is only slightly sensitive to

the shorter visible light rays and extremely sensitive to the ultra-violet rays, line 313 being the most distinct. This line is among the ones most effective in antirachitic qualities as proved by the work of Hausser and Vahle and shown in their chart on "Skin sensibility to ultra-violet rays."<sup>3</sup>

OTHER FABRICS TESTED

Many other cotton fabrics of different weights were tested, and in each case the ratio of protection secured by screening printing-out paper from the rays of the lamp proved the correct ratio for time of exposure to produce burns on the skin both in direct sunlight and under the lamp. This was also true of all linen fabrics tested. In all cases this ratio was smaller than the ratio of the interspace between the threads to the space covered by the threads.

Two of the wool fabrics included in the test required a ratio of 20 to 1 for time of exposure to produce a burn, as shown in Table 6, while the third piece of wool, which was very much lighter, required a ratio of only 10 to 1; this, it will be noticed, is slightly more than double the ratio obtained by ultra-violet light and the sensitized paper.

TABLE 6.—Coefficient of protection of additional textile fabrics

Fabric	Coefficient determined by—			
	Photometer	Measure- ment of interspace	Action of ultra-violet rays on sensitized paper	Action of sunlight on skin
True silk.....	3.606	13.60	4.00	5.00
Do.....	3.844	19.10	6.00	12.00
Wild silk.....	4.850	14.59	8.50	10.00
Do.....	4.270	10.27	7.50	9.00
Wool.....	4.863	11.90	8.50	20.00
Do.....	4.940	10.40	9.00	20.00
Do.....	3.920	11.64	5.00	10.00
Cotton.....	5.280	-----	3.00	3.00
Linen.....	3.360	4.04	2.33	2.33

The silk fabrics, of which four more were listed, two of wild silk and two of cultivated silk, gave varied results as a group. The individual fabrics gave the same results on repeated exposure, but no definite group results were obtained with silk as with the other fibers. In no case was the ratio of time of exposure necessary to secure a burn of the skin through silk less than the ratio of protection obtained by means of the lamp and the printing-out paper used.

Although they varied widely the ratios indicated roughly the time of exposure to be employed when subject was under test. The ratio of time of exposure obtained by means of lamp and sensitized paper proved correct for time of exposure for all linen and cotton fabrics tested, while in the case of silk and wool fabrics the ratio obtained by this method was in every case less than the ratio required to secure a good burn.

<sup>3</sup> HAUSSER, K. W., and VAHLE, W. DIE ABHÄNGIGKEIT DES LICHTERYTHEMS UND DER PIGMENTBILDUNG VON DER SCHWINGUNGSZAHL (WELLENLÄNGE) DER ERREGENDEN STRAHLUNG. Strahlentherapie 13: [41]-71, illus. 1922.

Tests proved that the skin burned in a shorter time of exposure to ultra-violet rays when the blood had been brought to the surface by application of heat. Such comparisons were obtained by exposing controls while the arm was still cool and comparing them with controls exposed after the arm had become warm from heat rays or from the application of heat to the arm.

#### CONCLUSIONS

The data submitted from a number of tests tend to prove that the protection from sunburn afforded the skin by fabrics depends primarily upon the per cent of interspace due to weave but that the vegetable fibers, cotton and linen, transmit some of the rays that burn and tan, thus offering a small coefficient of protection, whereas the animal fibers, silk and wool, absorb a larger portion of these rays, thus offering a higher coefficient of protection.

Due to the fact that temperature is a factor in the burning of the skin the greater conductivity of cotton and linen, as compared with that of silk and wool, might in a measure account for the higher prospective value of silk and wool.



# SOME NITROGENOUS CONSTITUENTS OF CORN POLLEN<sup>1</sup>

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## HISTORICAL

The first work recorded on the chemical analysis of pollen was by Fourcroy and Vauquelin (1),<sup>2</sup> who studied the ash and organic constituents of pollen of the date palm, *Phoenix dactylifera*. They found that this pollen contained a substance analogous to gluten, and a large amount of malic acid. Link (9, p. 145) reported much tannin and gluten in the pollen of the hazel tree. Vauquelin (9, p. 237) found sugar, resin, and tannin in stamens of the horse chestnut. Braconnot (6) reported the presence of potassium malate and a little nitrogenous material in the pollen of *Typha latifolia*.

Planta (35) found sucrose, starch, and 4.81 per cent nitrogen in the pollen of *Corylus avellana*. An extract of this pollen with 10 per cent NaCl solution gave a coagulum on heating, and peptones were also present. In the pollen of *Pinus sylvestris*, Planta (36) found 2.65 per cent nitrogen. Peptones, guanine, and hypoxanthine were present. An extract of the pollen with 10 per cent NaCl solution did not, however, give a coagulum on heating. Schulze and Planta (39) found a nitrogenous substance in the pollen of *Corylus* and pine. This substance yielded guanine and glucose, hence was called guanosin. Przybytek and Famintzin (37) found 2.4 per cent nitrogen in the pollen of *Pinus sylvestris*, and Kresling (25) reported tartaric and malic acid in the same kind of pollen.

Stift (41), working on pollen of the sugar beet, found the total nitrogen to be 3.6 per cent, protein nitrogen 2.66 per cent, and lecithin 1.57 per cent. Stoklasa (42) reported 5.16 per cent lecithin in pollen of the horse chestnut, and 5.86 per cent in that of the apple. Kammann (21) found that nitrogen containing organic substances made up 58 per cent of the total organic material of rye pollen, and of this 40 per cent was protein in nature and 18 per cent nonprotein. Winterstein and Hiestand (50) found in the pollen of *Alnus viridis* and *Pinus montana* a phosphatide which contained lecithin in combination with a complex carbohydrate.

Koessler (24) found the following nitrogen distribution in ragweed pollen:

	Per cent
Total nitrogen.....	4. 73
Nitrogen soluble in 5 per cent NaCl solution.....	1. 89
Nitrogen soluble in 5 per cent NaCl solution and coagulable....	. 10
Nitrogen soluble in 5 per cent NaCl solution and noncoagulable.....	1. 66
Nitrogen soluble in 75 per cent alcohol after 5 per cent NaCl extraction.....	1. 57
Nitrogen in the insoluble residue.....	1. 35

<sup>1</sup> Received for publication Mar. 14, 1927; issued September, 1927. Published with the approval of the director as Paper No. 675, Journal Series, Minnesota Agricultural Experiment Station. Condensed from a thesis presented by C. G. Vinson to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of doctor of philosophy, Ross Aiken Gortner, major adviser.

<sup>2</sup> Reference is made by number (*italic*) to "Literature cited," p. 275.

Heyl has done the most extensive work on the chemical analysis of pollen. He found (13) that the exine was removed largely by extracting with ether and then alcohol; but that this did not remove the intine which is largely cellulose. Extracting with ether in a Soxhlet apparatus removed 13.26 per cent of the solids, and alcohol following the ether extraction removed an additional 20.9 per cent; but hot 95 per cent alcohol alone extracted 42.9 per cent of the solids. The pollen contained 4.99 per cent nitrogen, of which 1.08 per cent was alcohol soluble, 2.6 per cent was soluble in 10 per cent NaCl solution, and 0.8 to 1.1 per cent was extracted by 0.2 per cent alkali after extracting with 10 per cent NaCl. The residue from the various extractions contained 1.2 per cent nitrogen. In other papers Heyl (16, 17) reports the nonnitrogenous constituents extracted by alcohol and ether. Ragweed pollen (14), which had been exhausted with ether and 95 per cent alcohol and then extracted with distilled water, gave 1.22 per cent albumin, coagulating at 65° C., and 1.1 per cent proteose. After extracting with distilled water and then with 10 per cent NaCl, dilute alkali extracted a protein which amounted to 2.9 per cent of the original material. This protein was precipitated on acidification, and was the chief protein present. The percentage of certain amino acids in these proteins has been reported by Heyl and Hopkins (18). Heyl (15) found that the coloring substances of ragweed pollen belong to the flavonols and are entirely glucosidic. Quercetin glucoside having the composition of  $C_{21}H_{20}O_{12}$  was found. A glucoside of isorhamnetin was also obtained.

Corn pollen, however, has not received so much attention as ragweed pollen. Miyake (28) found phytosterol and inosite in corn pollen. Miyake (29) later identified adenine and choline. Anderson and Kulp (3), working on corn pollen, found 4.3 per cent nitrogen in the air-dry pollen. Phytosterol palmitate was identified. Inosite was found to be present to the extent of 0.83 per cent, choline 0.34 per cent, and 1-proline to the extent of 0.6 per cent.

Anderson (2) found a saturated hydrocarbon, probably nonacosane, attached to the phytosterol palmitate from corn pollen. The phytosterol preparations differed, however, from ordinary phytosterol.

#### MATERIAL

Since no particular attempt had been made to identify the nitrogenous constituents of corn pollen, it was decided to concentrate on the nitrogenous fraction. About 4 kgm. of air-dry pollen, collected in 1917 and 1918, were available. The pollen was quite clean, as it had been screened through bolting-cloth, thus freeing it from anther sacs, filaments, etc. The air-dry pollen had a nitrogen content of 3.6 per cent and a moisture content of 3.97 per cent, as determined by bringing a sample to constant weight at room temperature in vacuo over  $H_2SO_4$ , as did Anderson and Kulp (3). Corn belongs to the anemophilous class of pollinated plants, and agrees with the findings of Lidforss (26) in that the nitrogen content of its pollen is relatively low as compared with pollen of entomophilous flowers.

According to Heyl (14), the walls (exine and intine) of the pollen grain constitute 65 per cent of the structure. The exine is relatively thick and resistant to mechanical disintegration. Planta (35) found that grinding pollen between grooved steel plates had no effect on

the grains. Winterstein and Hiestand (50) state that it is difficult to extract the entire grains, so part was crushed in a mortar and part ground in a mill. Anderson and Kulp (3) found that long grinding in a mortar, even after the pollen had been extracted with ether and alcohol, produces only a small percentage of broken cells. Realizing that a better extraction could probably be obtained with the pollen grains pulverized, the writer placed 313 gm. of the air-dry pollen in a ball mill of 4,000 c. c. capacity, and at the end of 24 hours grinding not an entire pollen grain could be found when a sample was examined under the microscope. After the ball mill had proved so efficient in pulverizing the grains, practically all the pollen was put through it.

#### PRELIMINARY EXPERIMENTS

Ten grams each of the milled and unmilled pollen were extracted with two 75 c. c. portions of ether in a Soxhlet apparatus, allowing 12 hours to each extraction. This was followed by extraction with two 150 c. c. portions of absolute alcohol in a 300 c. c. Florence flask, allowing 12 hours to each extraction. From the milled sample 3.0200 gm. of dry matter containing 0.0300 gm. of nitrogen were extracted, and from the unmilled 3.0250 gm. containing 0.0330 gm. of nitrogen, indicating that the unground pollen was as readily extracted by ether and alcohol as was the ground material.

Again 10 gm. each of fresh milled and unmilled pollen were extracted 14 hours in the cold with 150 c. c. of 5 per cent  $K_2SO_4$  solution. The filtered extracts were made up to volume and total nitrogen determined on aliquots. The extract from the unmilled pollen contained 0.1455 gm. of nitrogen and that from the milled sample contained 0.1765 gm. of nitrogen, showing that the nitrogen was more readily extracted from the milled than the unmilled pollen. Approximately 50 per cent of the nitrogen was extracted from the milled pollen by the  $K_2SO_4$  solution.

The residue of the unmilled pollen from the above extraction with 5 per cent  $K_2SO_4$  solution was extracted 15 hours at room temperature with 150 c. c. of 5 per cent  $H_2SO_4$ . After filtering and making the filtrate up to volume, it was found to contain 0.0525 gm. of nitrogen. The residue from this extraction was then treated with 150 c. c. of 0.2 per cent NaOH, but after mixing, the reaction was neutral to litmus. Accordingly, sufficient additional dilute NaOH solution was added to give an alkaline reaction to litmus. After extracting for 15 hours the material was filtered by suction, washed, and the filtrate made up to volume. The total nitrogen in this extract was found to be 0.0512 gm. Thus the total nitrogen successively extracted from a sample of unmilled pollen by 5 per cent  $K_2SO_4$ , 5 per cent  $H_2SO_4$ , and then dilute alkali was  $0.1455 + 0.0525 + 0.0512 = 0.2492$  gm. This is 2.49 per cent of the original air-dry pollen; hence the total nitrogen not extractable with aqueous solutions  $= 3.60 - 2.49 = 1.11$  per cent. Heyl (13) found 1.2 per cent nitrogen in the residue of ragweed pollen from similar extractions.

The residue from each of the above samples which had been extracted with ether and boiling absolute alcohol was extracted in the cold with 150 c. c. of 5 per cent  $K_2SO_4$  solution for 14 hours. On filtering and making the filtrates up to volume, that from the milled

sample was found to contain only 0.036 gm. of nitrogen and that from the unmilled sample 0.046 gm. Apparently the ether and boiling alcohol made the remaining nitrogen less soluble, and the more intimate contact of the ether and alcohol with the ground pollen rendered a greater amount of the remaining nitrogen insoluble than in the case of the unground pollen. This precluded the possibility of extracting the pollen with ether and boiling alcohol before extracting the nitrogenous fractions with aqueous solutions. Heyl (14), however, found that in the case of ragweed pollen exhaustion with ether and alcohol at ordinary temperatures did not render the remaining nitrogen less soluble.

Ten gm. of milled pollen were extracted 24 hours with 150 c. c. of distilled water, the pollen residue centrifuged off, and the supernatant liquid then filtered. The pollen residue was then treated with 75 c. c. of distilled water, centrifuged, and the washings filtered. The filtrates were combined, made up to volume, and found to contain 0.129 gm. of nitrogen. The 5 per cent  $K_2SO_4$  extraction therefore removed  $0.1765 - 0.1290 = 0.0475$  gm. more of nitrogen than the distilled water alone. Therefore, to determine if a globulin was present, one 10-gram sample of milled pollen was extracted 14 hours with 100 c. c. of distilled water and another 10-gram sample of the milled pollen was extracted for a like period with 5 per cent  $K_2SO_4$ . The extracts were centrifuged and then filtered. Each was gradually heated. An opalescence began to form at  $75^\circ C.$  and a slight coagulum appeared at  $80^\circ$ . On heating each to boiling under a reflux for an hour, then filtering, 0.0346 gm. of coagulum was obtained from the aqueous extract and 0.0556 gm. from the 5 per cent  $K_2SO_4$  extract. Five gm. of  $K_2SO_4$  were then added to the aqueous extract and the solution again heated to boiling under a reflux for one hour. Another coagulum weighing 0.0294 gm. was obtained, making a total of 0.063 gm. of coagulum from the distilled water extract, which coagulum contained 0.0056 gm. of nitrogen, while that from the 5 per cent  $K_2SO_4$  extract contained 0.0053 gm. of nitrogen. The distilled water had, therefore, removed as much of the heat-coagulable nitrogenous material as the 5 per cent  $K_2SO_4$ , and, therefore, the presence of a globulin was improbable, since the salt content of the pollen would hardly give a high enough concentration to hold globulins in solution. Heyl (14) found, however, that salt extraction removed more of the heat coagulable fraction from ragweed pollen than extraction with distilled water.

#### ENZYMES

On warm days when the hand came in contact with the pollen the small amount of perspiration on the fingers seemed to cause the pollen to partially autolyze and form a waxy paste. If this was a case of autolysis, then enzymes must have been present. Kammann (22) found proteose in rye pollen. Paton (32) working on the pollen of Easter lily, red maple, Norway maple, Siberian crab apple, Austrian pine, magnolia, dandelion, goldenrod, ragweed, and corn, found erepsin, pepsin, and trypsin in some. Trypsin and pepsin (33) were found in corn pollen. Hence it was quite necessary to determine whether proteolytic enzymes were present before attempting to isolate the nitrogenous fractions. In order to try to determine the presence of active enzymes, ten 5-gram samples of

the milled pollen were placed in 10 separate 250 c. c. Erlenmeyer flasks, and the flasks were then plugged with cotton and allowed to stand in an incubator at 35° C. overnight. The next morning 100 c. c. of distilled water, which had attained the same temperature by standing in the incubator overnight, were added to each of the 10 flasks, rotating the flasks to secure thorough wetting of the pollen, and 2 c. c. of toluene were added. The flasks were then agitated at half-hour intervals, except at night. The water was added at 7.30 a. m. The first flask was removed at 8 a. m.; the second at 10 a. m.; the third at 12.30 p. m.; the fourth at 3 p. m.; the fifth at 7 p. m.; the sixth at 8 a. m. the second day; the seventh at 4 p. m. the second day; the eighth at 8 a. m. the third day; the ninth at 8 a. m. the fourth day; and the tenth at 8 a. m. the fifth day. On being removed from the incubator each sample was made up to 150 c. c., 50 c. c. of which were used for the  $\text{SnCl}_2$  precipitation<sup>3</sup> and another 50 c. c. for the copper precipitation.<sup>3</sup> After precipitating the proteins in the two latter fractions with  $\text{SnCl}_2$  and copper reagents, respectively, each was made up to 100 c. c., centrifuged, filtered, and total nitrogen determined on 25 c. c. samples and amino nitrogen determined on 2 c. c. samples. The 50 c. c. remaining, after taking the samples for  $\text{SnCl}_2$  and copper precipitation, were filtered, and total nitrogen was determined on 25 c. c. samples and reducing sugars on 5 c. c. samples. Table 1 shows the data which were obtained.

TABLE 1.—Autolysis of corn pollen (5-gram samples)

Sample No.	Total nitrogen in the entire aqueous extract	Total nitrogen not precipitated by $\text{SnCl}_2$	Total amino nitrogen not precipitated by $\text{SnCl}_2$	Total nitrogen not precipitated by copper	Total amino nitrogen not precipitated by copper	Total reducing sugars
	Grams	Grams	Grams	Grams	Grams	Milli-grams
1.....	0.055	0.025	0.002	0.020	0.008	48.76
2.....	.056	.032	.006	.025	.006	65.76
3.....	.056	.036	.003	.036	.002	72.75
4.....	.059	.027	.000	.031	.000	77.51
5.....	.066	.027	.002	.039	.002	77.83
6.....	.064	.034	.005	.039	.001	80.05
7.....	.063	.025	.005	.043	.006	82.50
8.....	.062	.037	.006	.044	.004	85.77
9.....	.071	.033	.016	.039	.010	88.31
10.....	.075	.036	.016	.043	.016	91.17

The precipitation with copper as recommended by Olsen<sup>4</sup> was much less satisfactory than with the  $\text{SnCl}_2$  reagent, owing to the fact that the end point with phenolphthalein was obscured by the color of the solution. The same difficulty was also experienced in using bromcresol purple as an indicator in the  $\text{SnCl}_2$  precipitation.

The small amount of amino nitrogen present renders these determinations subject to great error, and perhaps of little value. It is evident, however, that the total nitrogen in the filtrates from the two precipitations did not increase to any appreciable extent after

<sup>3</sup> OLSEN, A. G. A STUDY OF THE PROTEASES OF BREAD YEAST, *SACCHAROMYCES CEREVISIAE*. [Unpublished master's thesis. Copy on file, Div. Agr. Biochem., Univ. Minn., St. Paul.] Part of the data in this thesis has been published (37).

<sup>4</sup> Olsen, A. G. Op. cit.

the first two hours. The total reducing sugars, however, show a steady increase, as might be expected from the reports of Van Tieghem (45), Erlenmeyer (10), and Green (12), working on the pollens of other species; hence carbohydrases were present and active.

From the above data it was evident that extractions of the pollen could be made with aqueous solutions without danger of alteration of the nitrogenous fractions by proteolytic enzymes.

#### PRELIMINARY SURVEY

For preliminary survey work, 500 gm. of the milled pollen were extracted 20 hours with 3,000 c. c. of distilled water. Fifteen hundred cubic centimeters of the water were first added, but after agitation and standing 15 minutes the entire mass set to quite a stiff gel. Davison (unpublished data) later found a very active pectase in this sample of pollen, which may explain the gel formation. Lloyd (27) has shown that the pollen tubes may show great variation in the amount of water which they will absorb. This may be associated with a gel formation.

The aqueous extraction was carried out by keeping the material, which was in a 5-liter bottle, on a shaking machine for eight hours. The remainder of the time the bottle was shaken at intervals. At the end of 20 hours the material was passed through a supercentrifuge. The filtrate was then filtered through paper pulp in order to clarify it. The solution was then carefully concentrated in vacuo, keeping the temperature below 50° C. This solution was acid to Congo red in reaction, and on attempting to coagulate the heat-coagulable proteins by boiling, only a slight turbidity developed. On testing for albumin by the method of Sørensen (40, p. 28) the turbidity increased only slightly. This indicated the presence of very little albumin in the corn pollen. Heyl (14), however, found 1.22 per cent of albumin in the aqueous extract of ragweed pollen.

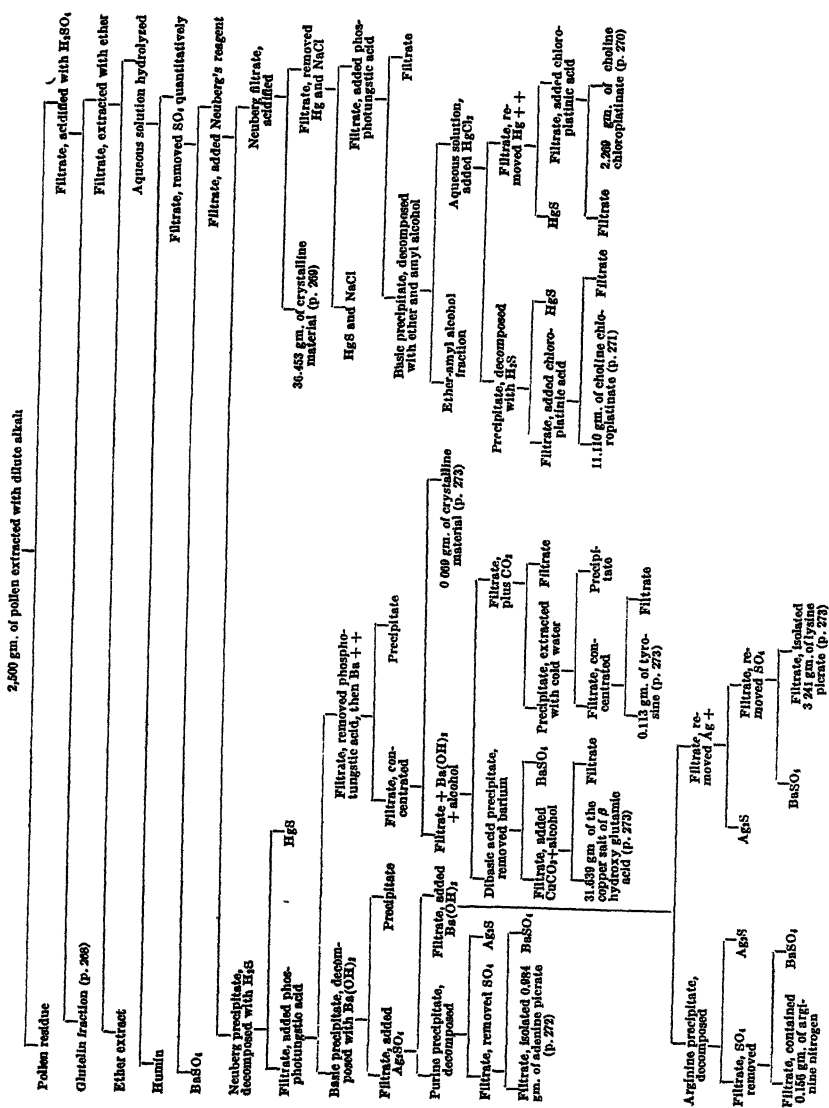
The pollen residue from the above aqueous extraction was extracted with 2,000 c. c. of 0.2 per cent NaOH solution for 24 hours, being kept on a shaking machine for eight hours. This material was likewise put through the supercentrifuge, as it was impossible to filter by gravity or suction. The ordinary centrifuge could not be used, as much of the fine material could not be thrown down with this type of centrifuge. The filtrate was dark in color and quite viscous. It was filtered, and a gelatinous precipitate filtered off. The reaction of the filtrate to litmus was neutral; so evidently not enough alkali had been used to keep the material in solution. The gelatinous precipitate was extracted with 3,000 c. c. of a 0.4 per cent NaOH solution, and on filtering only a negligible residue remained. The filtrate was very dark in color and on being made acid to litmus, but alkaline to Congo red, by carefully adding 5 N.H<sub>2</sub>SO<sub>4</sub> a gray-colored flocculent precipitate settled out, leaving a clear amber-colored supernatant liquid.

The gray precipitate was extracted 24 hours at room temperature with 1,000 c. c. of 5 per cent K<sub>2</sub>SO<sub>4</sub> solution, remaining on the shaking machine eight hours. The solution was filtered, and the filtrate plus the washings concentrated and made up to 1,000 c. c. It was found to contain about 0.100 gm. of nitrogen. If this latter extract represented globulin, certainly there was very little present in the pollen worked with. Heyl (14) found an albumin in ragweed pollen,

but does not report the presence of globulins. Bauman, Chudnoff, and Mackenzie (5), however, speak of the globulin fraction. The precipitate obtained on neutralizing the alkaline extract is termed the glutelin fraction.

## MAIN EXPERIMENT

## OUTLINE OF PROCEDURE



One kilogram of the ground pollen was placed in a 5-liter bottle and 3,000 c. c. of distilled water added. In using 0.2 per cent NaOH solution on the preliminary sample, the resulting solution was neutral to litmus; consequently, in this case, 15 per cent NaOH solution was added until the mixture gave an alkaline reaction to litmus, 200 c. c. of the alkali solution being required. The pollen was extracted 24 hours, being kept on a shaking machine for 10 hours. A like extraction was made of another kilogram sample, and this plus the 500 gm. used in the preliminary work made  $2\frac{1}{2}$  kgm. extracted. At the end of each extraction the material was passed through a supercentrifuge, and then the filtrates were combined. The filtrate from the above extractions was alkaline to litmus, but not to phenolphthalein. The pollen residue when suspended in 3,000 c. c. of distilled water again gave a neutral reaction to litmus. This suspension was made alkaline to litmus with NaOH solution, and allowed to stand 14 hours with shaking at intervals. At the end of the extraction the material was centrifuged as before.

Upon adding the second extract to the first the combined extracts gave an alkaline reaction to phenolphthalein. By carefully adding 5N.H<sub>2</sub>SO<sub>4</sub> the reaction was carried to that point where all material was yet in solution but precipitation would take place with only a slight amount of acid. The solution was then concentrated in vacuo at a very low temperature. The solution (No. 11) was made up to 8,000 c. c. and the following data obtained from its analysis:

	Grams
Total nitrogen.....	51.412
Ammonia nitrogen.....	.440
Amide nitrogen.....	1.840
Amino nitrogen.....	8.444
Organic solids.....	816.64

After aliquoting, 5N.H<sub>2</sub>SO<sub>4</sub> was slowly added to the remainder of the concentrated extract until it was just acid to Congo red, when a gray-colored precipitate formed. This precipitate was light, and tended to come to the top of the liquid. By adding ether and rapidly stirring the liquid it was found that the precipitate would form a uniform layer at the top from which it could be siphoned off into ordinary centrifuge tubes and a further separation obtained by centrifuging in the ordinary type of centrifuge.

After separating the glutelin precipitate in this fashion it was extracted with 2 liters of ether, to remove fat-soluble materials. The precipitate was then dissolved in 6 liters of slightly alkaline water, and reprecipitated by acidifying with acetic acid. The dry precipitate weighed 248.1 gm., contained 22.461 gm. of nitrogen and 19.97 gm. of ash.

The combined filtrates from the glutelin precipitation were concentrated in vacuo and extracted with 2 liters of ether. The aqueous solution was then concentrated, made up to volume (solution 12), aliquoted, and the following data obtained on analysis:

	Grams	Per cent of solution 11
Total nitrogen.....	25.790	52.68
Ammonia nitrogen.....	.320	72.82
Amide nitrogen.....	.721	39.18
Amino nitrogen.....	7.372	83.71
Organic solids.....	716.400	87.73

As one would expect, the greater amount of the amino nitrogen came down in the filtrate from the glutelin, and by difference the greater amount of the amide nitrogen remained in the glutelin fraction.

The remainder of solution 12 was hydrolyzed 24 hours with 20 per cent  $\text{H}_2\text{SO}_4$ . The humin from this hydrolysis weighed 49.22 gm. and contained 1.942 gm. of nitrogen. Sulphate was exactly removed with  $\text{Ba}(\text{OH})_2$ . After precipitation was complete the solution containing the  $\text{BaSO}_4$  was heated and kept almost at the boiling point for about two hours, then allowed to cool slowly. In this way the  $\text{BaSO}_4$  precipitated in relatively coarse particles, and the solution then filtered fairly readily and a relatively small amount of nitrogen was lost by adsorption. The  $\text{BaSO}_4$  weighed, when dry, 3,712 gm. and contained 0.989 gm. of nitrogen, which is a low figure for nitrogen in such a large amount of precipitate.

The filtrate and washings from the  $\text{BaSO}_4$  precipitate were concentrated, made up to volume (solution 13), aliquoted, and on analysis, gave the following:

	Grams
Total nitrogen.....	25.270
Ammonia nitrogen.....	1.477
Amino nitrogen.....	10.351
Organic solids.....	602.190

#### PRECIPITATION WITH NEUBERG AND KERB'S REAGENT

The remainder of solution 13 was concentrated, placed in a large precipitating jar, and precipitation with Neuberg and Kerb's (30) reagent carried out. Solutions containing 600 gm. of mercuric acetate and 215 gm. of  $\text{Na}_2\text{CO}_3$  were added until the volume reached 6 liters and no end point was indicated. It was then considered advisable to add an equal volume of alcohol, remove the precipitate, acidify and concentrate the filtrate, and repeat the Neuberg precipitation at greater concentration, as this reagent is supposed to act best when the amino acid concentration is 10 to 20 per cent. In the second precipitation 195 gm. of mercuric acetate and 60 gm. of  $\text{Na}_2\text{CO}_3$  were added, and still no orange-red precipitate was obtained on adding either of the two reagents. The failure to observe the characteristic colored precipitate at the end point may have been due to the color of the solution obscuring it, as in the first precipitation an orange-red precipitate collected on the stirring rod, and the last precipitate had a yellow-red layer on top. The last precipitate was very small. Both precipitations were carried out in the cold, the precipitating jar being surrounded by running water which was at a temperature of  $10^\circ \text{C}$ . In each case after adding the Neuberg reagent an equal volume of alcohol was added and the mixture then allowed to stand overnight. The two precipitates were combined and washed with 1,000 c. c. of 80 per cent alcohol. On acidifying the filtrate from the first precipitate with acetic acid, a flocculent precipitate formed which was filtered off and was found to be crystalline. It weighed 36.453 gm. and had a distinctly bright, metallic luster. It contained neither nitrogen nor sulphur. This substance is reserved for further investigation.

The filtrate from the last Neuberg precipitate was acidified, concentrated, and mercury was then removed as  $\text{HgS}$ . The filtrate and washings from the  $\text{HgS}$  were concentrated and  $\text{NaCl}$  removed

with concentrated HCl and alcohol. The filtrate from the NaCl was concentrated and made up to volume (solution 15), aliquoted, and the following figures obtained on analysis:

	Grams
Total nitrogen.....	11.127
Ammonia nitrogen.....	.669
Amino nitrogen.....	2.164
Organic solids.....	266.325

#### PRECIPITATION OF BASES FROM THE NEUBERG FILTRATE

Bases were precipitated from the remainder of solution 15 by adding 5 per cent by weight of HCl and then adding a 20 per cent solution of phosphotungstic acid in 5 per cent HCl until precipitation was complete. After standing in the cold overnight, the precipitate was filtered off, and washed with a 2.5 per cent solution of phosphotungstic acid in 5 per cent HCl. Phosphotungstic acid was then removed from the basic precipitate with amyl alcohol and ether according to the method of Van Slyke (44) and Jacobs (20). The precipitate was suspended in water in a large Erlenmeyer flask, acidified with concentrated HCl, a mixture of equal parts of amyl alcohol and ether was added, and the flask agitated. It was necessary to filter twice before obtaining a good boundary between the two liquids the first time. Three extractions with the amyl alcohol-ether mixture were sufficient to remove all phosphotungstic acid from the aqueous solution so that no precipitate was obtained on adding a drop of a saturated solution of Ba(OH)<sub>2</sub>. A small amount of the precipitate failed to go into solution. This was ground up with baryta and water and yet failed to go into solution. The alkaline extract thus obtained was very dark in color, and on being acidified gave a precipitate of colored material. It contained a total of 0.082 gm. of nitrogen. It is believed the insoluble part was largely humin.

#### BASES OF THE NEUBERG FILTRATE

The basic fraction as precipitated from the Neuberg filtrate (solution 24) with phosphotungstic acid contained 3.587 gm. of nitrogen, 0.2989 gm. of which was amino nitrogen, showing that only about 14 per cent of the amino nitrogen of the Neuberg filtrate was basic nitrogen. Schulze's (38) method was accordingly used to separate choline from other plant bases. Mercuric chloride in boiling aqueous solution was added to the remaining portion of solution 24 until no further precipitate was obtained (4), and the mixture was allowed to stand overnight in the cold before filtering. The precipitate was extracted with boiling water, the insoluble portion was filtered off, and the two filtrates were combined. Mercury was removed as HgS, and alcohol was added to the concentrated filtrate from the HgS. A precipitate weighing 8.849 gm. and containing 0.2159 gm. of nitrogen formed at this point. This consisted of a mixture of ammonium and sodium chloride. The filtrate from the above precipitate was made up to volume and on analysis was found to contain 1.825 gm. of nitrogen. To an aliquot of this solution a 5 per cent platonic chloride solution was added until precipitation was complete. The chloroplatinate obtained weighed 0.4538 gm. and contained 31.84 per cent platinum; hence it must have been choline chloroplatinate.

The insoluble mercury compound was suspended in water and decomposed with  $\text{H}_2\text{S}$ . The filtrate from the  $\text{HgS}$  was taken down to dryness on a water bath. The residue was dissolved in a little hot absolute alcohol and again concentrated to dryness in vacuo. Very large needle-shaped crystals formed. The crystals were dissolved in absolute alcohol with warming and made up to 250 c. c. (solution 33). This solution contained 1.005 gms. of nitrogen. To 50 c. c. of this solution platonic chloride was added until precipitation was complete. A total of 2.222 gm. of chloroplatinate were obtained from this aliquot, thus giving a total chloroplatinate obtainable from the solution of 11.110 gm. This chloroplatinate was found to contain 31.73 per cent of platinum; hence it must have been choline chloroplatinate as the theoretical platinum content for choline chloroplatinate is 31.68 per cent. From the betaine fraction, as precipitated from the Neuberg filtrate, a total of 13.379 gms. of choline chloroplatinate were thus obtainable, representing 0.608 gm. of nitrogen.

#### THE NEUBERG PRECIPITATE

The Neuberg precipitate was brought into suspension in water, acidified with acetic acid, and decomposed with  $\text{H}_2\text{S}$ . The filtrate and washings from the  $\text{HgS}$  were concentrated and made up to volume (solution 14), aliquoted, and on analysis found to contain:

	Grams
Total nitrogen.....	11.810
Ammonia nitrogen.....	2.058
Amino nitrogen.....	3.980
Organic solids.....	95.720

Following the use of Neuberg's reagent the ammonia nitrogen increased 84.63 per cent over that in solution 13, and the amino nitrogen decreased 40.64 per cent. In some similar work reported by Vickery and Vinson (48), following the use of Neuberg's reagent, the ammonia nitrogen increased 7.3 per cent and the amino nitrogen decreased 15.53 per cent. It is entirely possible that the poor results in precipitating amino nitrogen with Neuberg's reagent were due to adding an excess of the reagent; for mercury is known to be a very effective catalyst, and an excess of the mercuric acetate may bring about a decrease in the amino nitrogen by catalyzing deamination and ring formation.

#### BASIC FRACTION OF THE NEUBERG PRECIPITATE

Bases were precipitated by phosphotungstic acid from the fraction precipitated by Neuberg's reagent. The filtrate from the basic fraction, after the removal of phosphotungstic acid with  $\text{Ba}(\text{OH})_2$  and subsequent removal of the barium with  $\text{H}_2\text{SO}_4$ , was found to contain 4.292 gm. of nitrogen, 2.811 gm. of which were amino nitrogen. On finding the ratio of amino to total nitrogen so low, the solution was again hydrolyzed for 10 hours with 10 per cent by weight of  $\text{H}_2\text{SO}_4$ . After removing the  $\text{H}_2\text{SO}_4$  quantitatively the solution was found to contain 4.292 gm. of nitrogen, 3.505 gm. of which were amino nitrogen; thus, due to hydrolysis, the amino nitrogen was increased 24.7 per cent. Bases were again precipitated, and the precipitate obtained added to the main phosphotungstate precipitate, which was then decomposed with  $\text{Ba}(\text{OH})_2$ . The filtrate from the barium phos-

photungstate precipitate, after removal of barium was found to contain:

	Grams
Total nitrogen .....	3.274
Ammonia nitrogen .....	.291
Amino nitrogen .....	1.133
Organic solids .....	20.610

The ammonia nitrogen in the above was probably a volatile amine, in part at least, for in titrating the distillate after ammonia determinations an amine odor could be detected.

Separation of the bases was carried out as described by Vickery (46) adding  $\text{Ag}_2\text{SO}_4$  in boiling saturated aqueous solution until a brownish yellow precipitate was obtained, when a drop of the solution was added to a drop of saturated  $\text{Ba}(\text{OH})_2$  solution. The purine precipitate was decomposed with  $\text{H}_2\text{S}$ , and the filtrate from the  $\text{Ag}_2\text{S}$ , after removal of  $\text{SO}_4$ , was found to contain 0.317 gm. of nitrogen. Picric acid, in saturated aqueous solution, was then added until precipitation was complete. The dry weight of the picrate thus obtained was 0.984 gm., and its melting point was  $278^\circ\text{C}$ . (uncorrected). Picric acid was removed from the above picrate and the purine converted into the sulphate, as described by Vickery and Leavenworth (47). Crystals of the sulphate formed, but these were small. Picric acid was then added to the solution of the sulphate, and a picrate melting point  $281^\circ\text{C}$ . was obtained. This is the melting point of adenine picrate, and thus 0.199 gm. of nitrogen as adenine was accounted for.

#### ARGENINE FRACTION

The filtrate and washings from the purine precipitate were saturated with finely divided baryta. A buff-colored precipitate formed. The precipitate was decomposed with  $\text{H}_2\text{S}$ , and  $\text{H}_2\text{SO}_4$  in the filtrate from the  $\text{Ag}_2\text{S}$  was exactly removed. The filtrate from the  $\text{BaSO}_4$  was found to contain 0.546 gm. of nitrogen. Arginine nitrogen in this fraction was determined by the method of Van Slyke (43), using the apparatus devised by Holm (19). This gave an arginine nitrogen content of 0.156 gm.

#### LYSINE FRACTION

Silver was removed as  $\text{Ag}_2\text{S}$  from the filtrate and washings from the arginine fractions. Sulphate was then removed quantitatively with  $\text{Ba}(\text{OH})_2$ . The filtrate from the  $\text{BaSO}_4$  was found to contain 2.245 gm. of nitrogen. Lysine was precipitated from this solution by the method of Winterstein (49). The precipitate of the mercury salt thus obtained was centrifuged off and the filtrate found to contain only 0.202 gm. of nitrogen. The mercury salt was decomposed with  $\text{H}_2\text{S}$ , after being suspended in dilute  $\text{H}_2\text{SO}_4$ . The sulphate ion was then removed quantitatively, and the filtrate from the  $\text{BaSO}_4$  concentrated to small volume, when small, yellow, rectangular crystals formed in abundance. Chlorides were then removed quantitatively with  $\text{Ag}_2\text{SO}_4$ , the excess silver removed as  $\text{Ag}_2\text{S}$ , and  $\text{H}_2\text{SO}_4$  in the filtrate from the  $\text{Ag}_2\text{S}$  exactly removed. The filtrate and washings from the  $\text{BaSO}_4$  were found to contain 1.797 gm. of nitrogen. This was concentrated to small volume, brought to the sensitive point with absolute alcohol, and a saturated solution of picric acid in ab-

solute alcohol was added until precipitation was complete. A total of 3.241 gm. of lysine picrate decomposing characteristically at 250° to 253° C. were obtained. This accounts for 0.254 gm. of nitrogen.

#### NONBASIC FRACTION OF THE NEUBERG PRECIPITATE

Phosphotungstic acid was removed from the filtrate of the bases from the Neuberg precipitate. The filtrate and washings from the barium phosphotungstate were concentrated and SO<sub>4</sub> removed quantitatively. The filtrate (solution 19) from the BaSO<sub>4</sub> on analysis was found to contain the following:

	Grams
Total nitrogen.....	3.209
Ammonia nitrogen.....	0.042
Amino nitrogen.....	2.491
Organic solids.....	27.379

The remainder of solution 19 was concentrated to small volume, when 0.069 gm. of crystalline material separated. Under the microscope the crystals were found to be large needles which were not collected in sheaves, as in the case of tyrosine, and, furthermore, the crystals were lemon yellow in color, and, dissolved in alkaline solution, gave a light green solution. The melting point was 348.8° C. (corrected). The melting point approximates that of apigenin or myricetin (34) but the other physical properties do not correspond. Some of the material was fused with sodium, and the Prussian-blue test found to be negative as well as the test for reduced sulphur with sodium nitroprusside. The crystals were only slightly soluble in acid, but were soluble in alcohol. It is believed that this material is a flavone or flavonol.

#### DIBASIC ACID FRACTION

The filtrate from the 0.069 gm. of crystalline material was concentrated to a sirup and dibasic acids removed by the method of Foreman (11), but varied according to the method of Kingston and Schryver (23), precipitating the dibasic acids as the barium instead of the calcium salts. The dibasic acid fraction so precipitated, after removal of barium contained 2.311 gm. of nitrogen. Beta hydroxy glutamic acid (7, 8) was isolated from this fraction as the copper salt, precipitating it from aqueous solution with alcohol. On analyzing this salt the following data were obtained:

	Per cent	Percentage calculated for $\beta$ OH glutamic acid
Copper content found.....	27.78	28.3
Total nitrogen found.....	6.50	6.23
Amino nitrogen found.....	6.17	6.23

A total of 31.639 gm. of the copper salt were actually isolated. This contained 1.972 gm. of nitrogen, corresponding to a weight of 22.96 gm. of the  $\beta$  hydroxy glutamic acid, which is 0.92 per cent of the original (2,500 gm.) pollen sample.

#### MONOAMINO-MONOCARBOXYLIC ACID FRACTION

Carbon dioxide was passed into the alcoholic filtrate from the precipitate of the barium salts of the dibasic acid fraction. This was carried out at 0°C., according to the directions of Kingston and Schryver (23). From the carbamino precipitate soluble in cold

water, 0.113 gm. of crystals in characteristic sheaves were obtained. The crystals gave Millon's reaction and contained 7.64 per cent nitrogen. These crystals were, therefore, tyrosine, as the theoretical nitrogen content of tyrosine is 7.74 per cent.

#### DISCUSSION

The heat-coagulable fraction of the aqueous extract from the pollen contained only about 10 per cent of nitrogen, whereas albumin is generally considered to contain about 16 per cent. Likewise, the glutelin fraction contained scarcely 10 per cent of nitrogen. Accordingly, these fractions are either highly contaminated with nonprotein material or else belong to the glycoproteins.

It is evident that corn pollen contains very little, if any, globulin. Extraction with salt solution failed to give any more heat-coagulable nitrogen-containing material than extraction with distilled water; and extraction of the glutelin fraction with salt solution gave only a negligible amount of nitrogen.

The use of Neuberg's reagent in precipitating amino nitrogen proved quite disappointing. This is the second experience in which this reagent has seemed to be instrumental in decreasing amino nitrogen, probably catalyzing ring formation. In clear solutions where the end point is easily observed, this reagent no doubt can be satisfactorily employed; but it is very evident that under some conditions it is not at all satisfactory.

Since Kingston and Schryver have shown that the barium salt of inactive aspartic acid is quite insoluble, it would seem more advisable to remove the dibasic acid fraction before precipitating the bases with phosphotungstic acid; for on making the filtrate from the bases alkaline to phenolphthalein with  $\text{Ba}(\text{OH})_2$  the inactive aspartic acid may be lost, thus accounting for some of the nitrogen in the barium phosphotungstate precipitate. Immediate removal of the dibasic acids after hydrolysis would seemingly be preferable, for with the strong acids and bases present, in concentrating to small volumes, some interaction is inevitable. This viewpoint is supported by the fact that on hydrolyzing the second time the amino nitrogen was increased 24.7 per cent, and another basic precipitate was obtained on adding phosphotungstic acid. With the early removal of the relatively strong dibasic acids the tendency for amino nitrogen to decrease would surely not be so great, especially when using Neuberg's reagent.

The 0.069 gm. of crystalline material (p. 273) is believed to be a flavone or flavonol pigment. This conclusion is drawn from the following facts: It is lemon yellow in color; nitrogen and sulphur are absent; the melting point is high; it changes color in alkaline solution; and is soluble in alcohol, but quite insoluble in acid or neutral aqueous solutions.

Beta hydroxy glutamic acid is the first dibasic amino acid ever reported as occurring in pollen of any kind. Planta states that asparagin could not be detected in the pollen of *Corylus avellana*. Schulze and Planta were unable to detect asparagin or glutamin in the pollen of *Corylus* or pine. Heyl, working with ragweed pollen, was unable to get copper salts to separate from the amino fraction after removal of bases. The dibasic acid fraction gave a very high

yield of  $\beta$  hydroxy glutamic acid as 85.33 per cent of the nitrogen of this fraction as accounted for in the copper salt actually isolated. Aspartic and glutamic acids were absent.

#### SUMMARY

A systematic study of the nitrogenous compounds contained in the alkali extract of corn pollen, and not precipitated on neutralization is reported.

The pollen was effectively pulverized by grinding in a porcelain ball mill.

The air-dry pollen contained 3.97 per cent of moisture and 3.6 per cent of nitrogen.

Successive extractions with distilled water, 5 per cent  $K_2SO_4$ , dilute  $H_2SO_4$ , then dilute alkali removed nitrogen to the extent of 2.49 per cent of the pollen, leaving 1.11 per cent nonextractable by the above solvents.

A substance behaving like a glutelin was isolated and was found to make up 9.9 per cent of the air-dry pollen. It contained 24.96 per cent of the total nitrogen in the air-dry pollen.

Proteolytic enzymes were not active, but active carbohydrases and a pectase were present.

Twenty-five hundred grams of air-dry pollen were extracted with dilute alkali and the filtrate neutralized with dilute  $H_2SO_4$ , when a precipitate formed. This precipitate is termed the glutelin fraction. The filtrate from the glutelin fraction, after extraction with ether, was hydrolyzed and the following substances determined:

a. 0.985 gm. of adenine picrate containing 0.199 gm. of nitrogen was isolated.

b. Arginine nitrogen was determined by the method of Van Slyke. This gave 0.156 gm. of nitrogen as arginine.

c. 3.241 gm. of lysine picrate were isolated.

d. 0.1129 gm. of tyrosine was obtained.

e. 31.639 gm. of the copper salt of  $\beta$  hydroxy glutamic acid were isolated. Aspartic and glutamic acids were not present.

f. 13.379 gm. of choline chloroplatinate were obtained, containing 0.608 gm. of choline nitrogen.

0.069 gm. of what is believed to be a flavone or flavonol was isolated.

On acidifying the Neuberg filtrate with acetic acid 36.453 gm. of a crystalline substance with metallic luster were obtained. This substance contained neither nitrogen nor sulphur, and is believed to be the mercury salt of the above flavonol. The identification of this compound is reserved for a later report.

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# UTILIZATION OF THE GRAIN IN KAFIR AND CANE SILAGE BY DAIRY COWS<sup>1</sup>

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## INTRODUCTION

Throughout the Southwest and along the southern border of the Corn Belt a large acreage of the sorghums is grown and used as silage in feeding dairy and beef cattle. During the course of a feeding experiment with dairy cows at the Oklahoma Experiment Station, it was observed that a considerable quantity of the grain in such silages is passed out into the manure apparently unused. Because of this loss many farmers practice heading these crops before ensiling them, grinding the heads separately, and feeding them in the concentrate portion of the ration. There is a question as to whether the loss of grain from the silage is sufficient to justify this practice being more widely recommended.

## HISTORICAL

Cave and Fitch<sup>2</sup> found that as high as 90 per cent of the seeds in sorgo (sweet sorghum) silage pass through the cow undigested. The determinations were made by counting the number of seeds in aliquot samples of silage and of manure secured from two cows over a five-day period. As the result of this brief work these investigators raised the question as to whether it might not be advisable to remove the heads before ensiling this crop, and to use the ground heads in the grain ration.

In a feeding trial conducted by Aicher and McCampbell,<sup>3</sup> it was found that kafir and cane silages fed to steers in combination with two pounds of cottonseed meal daily had comparative feeding values as follows:

	With heads	Without heads
Kafir silage.....	72. 40	45. 31
Cane silage.....	69. 90	60. 60

Thompson<sup>4 5</sup> found after four years of feeding trials with swine that whole kafir was utilized less efficiently than ground kafir, and that these had relative feeding values on a numerical basis as follows:

	Whole kafir	Ground kafir
Self-fed.....	110	130
Hand-fed.....	100	111
Soaked, whole.....	87	
In the head (dry).....	86	

<sup>1</sup> Received for publication Apr. 25, 1927; issued September, 1927.

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<sup>6</sup> Information to the authors. 1927.

He concluded that the feeding value of cane seed and of the grain sorghums was increased 10 to 25 per cent by grinding, since when fed in ground form a larger proportion of the grain was prevented from passing through the digestive tract apparently unused.

#### EXPERIMENTAL METHODS

Eight dairy cows were divided into two groups, one receiving kafir silage and the other cane silage. The cows were fed individually, each animal being allowed 30 pounds of silage and 10 pounds of alfalfa hay per 1,000 pounds live weight. The grain ration of corn meal, ground oats, wheat bran, and cottonseed meal was fed according to the milk and butterfat yields to meet the calculated requirements of the Morrison feeding standard. Refused feeds were re-weighed and recorded.

A 20-day preliminary feeding period preceded the 10-day experimental period. During the latter period manure was collected from each cow daily. The cows remained in stanchions except when released for exercise, at which times an attendant was with them to collect all manure voided.

The cane and kafir grains voided by the individual cows were mechanically separated daily from the entire quantity of manure, by the use of screens and water. These recovered grains were dried, screened, fanned, and hand picked to remove all foreign material. Samples of these air-dry kernels were preserved for chemical analysis.

The grain from large quantities of cane and kafir silages was secured mechanically, by the use of sieves, water, and a fan to free the grain from all stalk and leaf material.

The dry weights of 1,000 hull-free kernels from the silage and from the manure were taken to determine whether any losses in weights of individual kernels had occurred during their passage through the cow's digestive tract.

Chemical analyses were made of the dry hull-free samples of grain obtained from the silage and from the manure, to determine by comparison what losses of nutrients had occurred in the kernels thus recovered from the manure.

#### PRESENTATION OF DATA

From 565 pounds of cane silage, 21 pounds of clean, air-dry grain were obtained. The air-dry grain constituted 3.72 per cent of the weight of fresh silage. Likewise, 611 pounds of fresh kafir silage contained 18.76 pounds of air-dry kafir grain, or 3.07 per cent of the weight of fresh kafir silage.

The first group of four cows consumed a net total of 1,374 pounds of cane silage, while the second group of four cows ate 1,364 pounds of kafir silage. One cow in the latter group was off feed. Exclusive of this animal, 1,096 pounds of kafir silage were consumed by the group. The first group consumed 51.11 pounds of grain in the form of whole cane seed in the silage, and of this amount 17.33 pounds, or 33.91 per cent, were recovered from the manure. Excluding the cow in the second group which was off feed, 33.65 pounds of kafir grain were consumed, of which 16.64 pounds, or 49.46 per cent, of kafir grain were found to have been voided in the manure. Data for the individual cows are presented in Table 1.

TABLE 1.—Losses of grain into the manure, from cane and kafir silage consumed by dairy cows

Cow No.	Cane silage group				Cow No.	Kafir silage group			
	Silage consumed	Calculated grain in silage	Grain recovered from manure	Loss		Silage consumed	Calculated grain in silage	Grain recovered from manure	Loss
	Pounds	Pounds	Pounds	Per cent		Pounds	Pounds	Pounds	Per cent
1.....	300	11.16	3.60	32.26	5 <sup>a</sup> .....	268	8.23	2.41	29.25
2.....	360	13.39	4.83	36.08	6.....	340	10.44	6.09	58.35
3.....	360	13.39	4.65	34.70	7.....	420	12.89	6.65	51.57
4.....	354	13.17	4.25	32.29	8.....	336	10.32	3.90	37.84
Total.....	1,374	51.11	17.33	33.91	Total, excluding cow 5.....	1,096	33.65	16.64	49.46

<sup>a</sup> Cow 5 refused to eat a part of the kafir silage for three days previous to and for four days during the experimental period. The manure voided, therefore, represents less than 268 pounds of kafir silage consumed during the 10-day period in which manure was collected.

The question naturally arose as to whether the cows had derived any benefit from the whole grain voided in the manure. By weighing 1,000 kernels, dried to constant weight, as obtained from silage and from the manure, it was found that the kafir grain had decreased 2.98 per cent in weight. Likewise the cane seed showed a decrease of 7.85 per cent in the weight of an equal number of kernels. All weights were of hull-free kernels.

Chemical analyses were made of the hull-free kernels to ascertain if possible what changes had taken place in the kernels during their passage through the digestive tract. The kafir and cane kernels recovered from the manure were found to have a greater ash content than those from silage, due possibly to absorption of certain salts excreted into the alimentary tract. The ether extract was decreased about one-fifth. Crude fiber, contained largely in the outer covering of the kernel, was reduced slightly in percentage in the kafir sample, but for some unexplained reason appeared to increase in the sample of cane seed. Perhaps this increase may be attributed to a somewhat greater loss of the contents of the cane seed. A very small loss in crude protein was observed in the cane and kafir kernels. The nitrogen-free extract was not significantly affected. The character and extent of these changes may be noted in Tables 2 and 3.

The proportion of hulls and the closeness with which they clasp the kernels varies with cane and kafir. The percentage of kafir-grain hulls obtained from the silage was 13.75 per cent and was reduced to 0.50 per cent as recovered from the manure. Likewise, the percentage of hulls on the cane seed was reduced from 5.77 to 4.20 per cent by passage through the cow's digestive tract.

TABLE 2.—Composition of hull-free cane and kafir kernels as obtained from silage and from manure

Sample	Moisture	Ash	Crude protein	Crude fiber	Nitrogen-free extract	Ether extract
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
Kafir grain:						
From silage.....	10.25	0.81	9.88	1.66	74.17	3.23
From manure.....	10.40	1.54	9.36	1.44	74.61	2.65
Cane seed:						
From silage.....	10.56	.60	8.40	2.11	74.83	3.50
From manure.....	9.44	1.43	8.35	2.88	75.06	2.84

TABLE 3.—*Changes in composition of dry matter in hull-free cane and kafir kernels which occurred during passage of the silage through the digestive tract of the cow*

Item	Kafir kernels		Cane kernels	
	Actual change (grams)	Percentage change	Actual change (grams)	Percentage change
Crude protein.....	-0.60	-5.45	-0.17	-1.81
Ether extract.....	-.65	-18.06	-.78	-19.94
Nitrogen-free extract.....	1.24	1.50	.78	.94
Crude fiber.....	-.24	-13.04	.83	35.32
Ash.....	.82	91.11	.90	134.33
Total weight.....	-.436	-2.98	-1.002	-7.85

## DISCUSSION OF RESULTS

The data presented show that approximately one-third of the cane seed and over two-fifths of the kafir grain in silage made from these plants, as used in this feeding experiment, were voided in the manure. A comparison of the chemical analyses of the grain from the silage and of that from the manure shows that a negligible quantity of the nutrients in these undigested kernels was utilized. The increased percentage of ash may be explained by the absorption of salts by the grain while in the digestive tract of the cow.

The losses of whole kernels were sufficient to raise the question again as to the desirability of heading cane and kafir before ensiling these crops. If the heads are first removed, they may be ground and added to the grain ration. If they are not removed but are fed in the silage, pigs or poultry may be given access to the manure to salvage a part of the lost grain.

The cost of labor and time, the facilities available, as well as the value of kafir grain as feed, are factors to be considered in deciding whether to head cane and kafir before putting these crops into the silo.

## SUMMARY AND CONCLUSIONS

When dairy cows were fed cane and kafir silage made from fairly mature whole plants, one-third of the cane seed and over two-fifths of the kafir grain were voided in the manure.

Chemical analyses showed little utilization of nutrients from these whole kernels during passage through the cow's digestive tract. Some ether extract was digested, whereas only a small percentage of the crude protein was utilized. The effect upon the crude fiber was variable.

Heading cane and kafir silage before ensiling these crops is recommended. Such a practice will depend upon several economic factors, such as labor, facilities, and the value of kafir grain as feed.

When cows are fed silage made from the entire plant, the whole grain which passes out into the manure may be salvaged by pigs or poultry.

# THE RELATION BETWEEN THE VITAMIN B CONTENT OF THE FEED EATEN AND OF THE MILK PRODUCED <sup>1</sup>

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## INTRODUCTION

It has been clearly demonstrated by investigators in England (1, 3)<sup>3</sup> and America (5, 7, 8, 9, 10) that the degree of potency of vitamins A and C in milk is largely dependent on the diet of the cow. The greater part of the evidence has been obtained from comparisons of milk produced on summer pasture with milk produced on the usual winter ration, consisting of silage, grain, and hay.

It would appear reasonable to expect also that the vitamin B growth-promoting factor in milk would be dependent on the diet of the cow. Evidence obtained by several investigators does not bear out such assumption in all instances. Osborne and Mendel (11) have reported that from the standpoint of vitamin B potency milk from pasture-fed cows was not superior to that from cows receiving winter rations. Later investigations were conducted by Kennedy and Dutcher (10), Hughes, Fitch, and Cave (9), who concluded that the presence of vitamin B in milk was dependent on the presence of this factor in the diet of the cow. More recently Hart (6) has expressed the opinion that milk produced by cows on pasture is no richer in vitamin B than that from cows fed the usual winter ration of grain, hay, and silage. It has also been reported that it is not possible through the feeding of excessive quantities of wheat germ to increase the vitamin B content of cow's milk (12, p. 18). Just recently Bechdel, Eckles, and Palmer (2) concluded that the feeding of cows on a ration deficient in vitamin B slightly reduced the vitamin B content of the milk. As the ration fed the cows used in this investigation was unpalatable and was not eaten in amounts sufficient for satisfactory nutrition, the authors were reluctant to consider the results as being highly significant.

For the past three years one of the authors has been in charge of an investigation to determine the vitamin B requirements of calves. Conclusive evidence has been obtained to show that a calf will grow normally to maturity, and produce normal offspring on a ration that carries an insufficient amount of vitamin B to support growth and well-being in rats (2). Since the animals used in these investigations had been fed on such a ration for over two years at the time of freshening, an excellent opportunity was afforded

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<sup>2</sup> The authors wish to acknowledge their appreciation of the helpful advice received from Prof. R. A. Dutcher in planning and carrying out the experiment.

<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 287.

for a study of the vitamin B content of milk which was produced on a ration definitely known to be deficient in vitamin B.

In all of the investigations previously carried out as cited above, the vitamin B content of the ration fed to the cows was not definitely determined through rat-feeding trials. Furthermore, in the previous investigations, the deficient ration was not fed for such a prolonged period of time before the cows began producing milk.

#### EXPERIMENTAL PROCEDURE

The object of the present study was to determine through laboratory feeding trials with rats, the vitamin B potency of milk produced by three of the vitamin B growth experimental animals (2), Nos. 1168, 1169, and 1170. Two of these cows, Nos. 1169 and 1170, had received the ration deficient in vitamin B (found so by 11 rat-feeding trials) throughout their growth and reproductive careers, a period of about 750 days. The other cow, No. 1168, had received a vitamin B supplement to her ration for a part of the time during her growth period (2, p. 424). Continued feeding of this vitamin B supplement (marmite yeast) was found unnecessary for growth and well-being, and it was dropped from the ration almost 300 days before she freshened.

Animal No. 1169 freshened December 18, 1925, and No. 1168 a week later. Two weeks after the latter had freshened, about 10 quarts of her milk and 30 quarts from No. 1169 were saved from 4 successive milkings for the experiments. The milk from these heifers was mixed, poured in sterile containers, sealed, and frozen solid. A refrigerator maintained at about 10° F. below zero was used for storing the milk until it was needed for experimental feeding.

The milk was all collected at one time for the reason that there was some doubt as to whether the heifers would be able to lactate on the experimental ration for any great length of time. It was fortunate that such provision was made, since it developed a few days later that the experimental ration was inadequate to meet the heavy demands of lactation. Subsequent investigations, the details of which will be submitted in a later paper, established definite proof that the apparent deficiency was not due to an inadequate supply of vitamin B. Since vitamin B storage has not been found in other species (13), it would have been very interesting to study this phase of the problem on cattle. The authors appreciated, however, that the possibility of vitamin B storage in these heifers was almost negligible, and also that the fact that the milk was obtained early in lactation would not materially depreciate the value of the data.

Animal No. 1170 freshened February 6, 1926, after it had been fed the deficient ration 776 days. Her milk was sampled and stored for experimental feeding as described above.

The experimental ration fed to the heifers was made up of dried sugar-beet pulp as the sole source of roughage and the following concentrates: Corn gluten, pearled hominy from white corn, polished rice, cornstarch, imported casein (Argentine), cane sugar, bone

meal, and a mineral mixture. Cod-liver oil was fed daily to supply fat soluble vitamins.<sup>4</sup>

The laboratory methods previously employed for vitamin studies by Dutcher and his associates (4) were used in this investigation. A vitamin B free basal ration (4, p. 383) was fed in connection with the milk, as the sole source of vitamin B supply. Twelve cubic centimeters of milk from cows receiving good winter rations when fed in this way had been found necessary to support normal growth. The experiments were planned, therefore, with five groups of rats in order to study daily allowances per rat of 8, 10, 12, 16, and 20 c. c. of milk. Twelve rats were allotted to each group at the beginning of the experiment, and the mixed milk from cows Nos. 1168 and 1169 was fed to them. Beginning with the ninth week of feeding, all of the rats but the group allowed 20 c. c. of milk daily were fed milk from heifer No. 1170, as the remaining supply from the other heifers was inadequate to complete the experiment.

The rats were put on experiment at an average age of 24 days. Their average initial weight was 37 gm. In all of the work individual cages with screen bottoms (3 meshes to 1 inch) were used, a practice which has been rigidly carried out in this laboratory for over three years. Iodine was fed in the drinking water. The milk was fed in a container separate from the basal ration, and two drops of cod-liver oil were added to the milk daily to insure an adequate supply of vitamins A and D.

#### DISCUSSION

The experimental data were averaged and put in graphic form as presented in Figure 1. The groups fed 8 and 10 c. c. of milk (curves A and B) deviate markedly from the normal. None of the individual rats in the group allowed 8 c. c. were normal in size at the end of 91 days of feeding, although every individual continued to gain in weight during the entire feeding period. Similar comment may be made also concerning the group allowed 10 c. c. of milk, except that one individual was normal in size at the end of the experiment. There was apparently no tendency toward pathological conditions in any of the rats of the two groups above mentioned or of the groups fed larger quantities of milk.

The group receiving 12 c. c. of milk (curve C or curve H) made much better growth than those receiving less. Nine rats, or 50 per cent of this group, were of normal or larger size at the end of 84 days of feeding.

When the feeding of milk from heifer No. 1170 was started as mentioned above, an additional group of six rats was put on an experiment in which 12 c. c. of milk was added to the basal ration. This was done in order to determine whether the growth of the young, more rapidly growing animals would indicate any difference in the milk from another cow on the same diet. No detectable differences in the growth of any of the groups of rats was observed when the milk was changed. However, the growth of the additional six animals conformed closely to that of the rats from the same stock

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<sup>4</sup>Details concerning the ration and feeding technic may be found in the *Journal of Dairy Science* (2, p. 47).

that were fed by Dutcher, Francis, and Combs (4) on 12 c. c. of raw milk from the college herd. For convenience of study, a copy of the growth curve on the latter data has been placed on the chart (curve F) beside those of the two groups of rats fed 12 c. c. of raw milk in this experiment (curves G and H). (Curve F copied from Dutcher, Francis, and Combs.)

In order to have enough animals of one age and weight to start the five groups at one time, the rats used were purchased from a supply company. It so happened that they were of a strain of a somewhat lighter weight than the laboratory stock. It is entirely probable that their growth (curve H) is as near normal for their weight as that of the Pennsylvania State Stock (curve G). From the evidence presented by this additional group in connection with

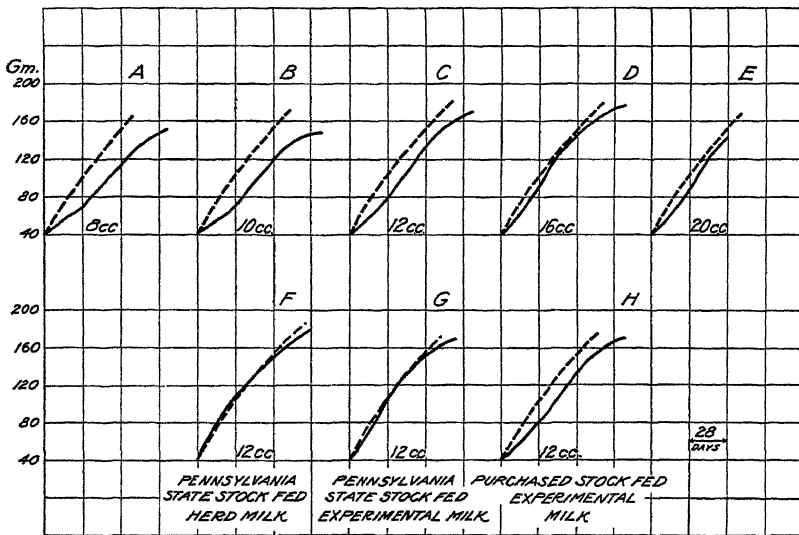


FIG. 1.—Growth curves. Broken lines represent normal growth of rats. Unbroken lines represent average growth of groups of rats fed different amounts of the experimental milk (excepting curve F, as indicated) as a supplement to a basal ration.

that of the original 12 rats, it is apparent that 12 c. c. of milk from cows on a ration deficient in vitamin B carried as much vitamin B as 12 c. c. of milk from cows receiving good winter rations.

The groups of rats allowed 16 c. c. (curve D) and 20 c. c. (curve E) of milk were so nearly identical that the experiment with the latter was discontinued after 56 days in order to save the milk for further feeding. Had the rats in these groups been of the Pennsylvania State stock their growth curve would no doubt have been above the Donaldson normal curve which is used in presenting all data from this laboratory. Furthermore, had the Pennsylvania State stock been used in the groups fed 8 and 10 c. c. of milk, the growth curves would have approached more closely to the normal.

The authors have for some time been conducting experiments to determine the possible synthesis of vitamin B by bacterial fermentations in the rumen of the digestive tract, as mentioned in an earlier

paper (2). Although these experiments are not completed, it may be said at this writing (April, 1927) that some very positive evidence has been obtained.

### SUMMARY

This investigation was conducted to determine the vitamin B potency of milk from three cows that were fed for over two years, throughout their growth period, on an experimental ration that was decidedly deficient in vitamin B, growth factor. The milk was fed to rats as a supplement to a vitamin-B-free basal ration on levels of 8, 10, 12, 16, and 20 c. c. per rat per day. The vitamin B potency of the milk was found equal to that of herd milk from cows receiving a good winter ration. It is concluded that vitamin B in milk is not dependent upon the presence of this vitamin in the ration of the cow. It would appear that cattle, and possibly all ruminants, differ from other animals in their ability to grow to maturity, to produce normal offspring, and to maintain vitamin B in their milk when forced to subsist on rations deficient in vitamin B. If this is true, it would appear that ruminants possess the power to synthesize vitamin B. Experiments are in progress to determine whether this synthesis is due to microorganisms normally present in the rumen.

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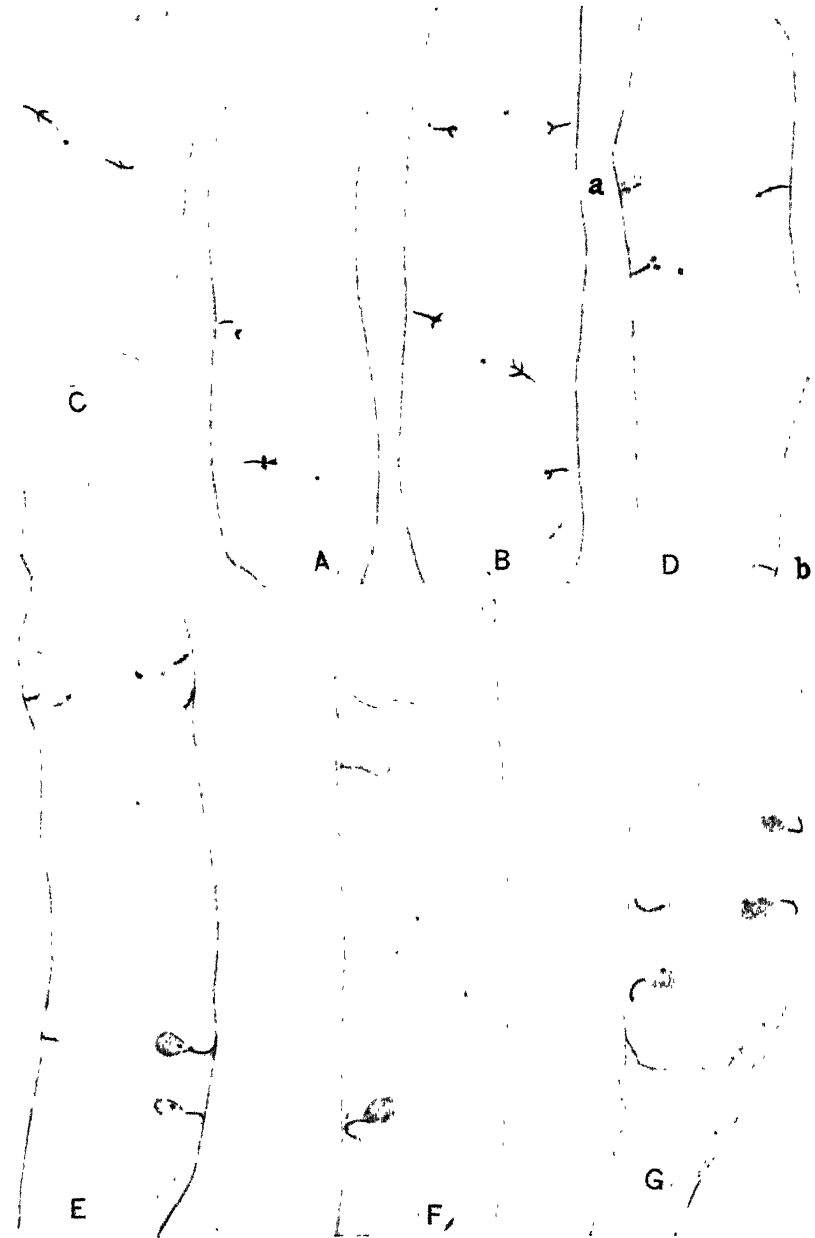
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(For explanatory legend see p. 290)



(For explanatory legend see p. 291)

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## NUCLEAR PHENOMENA ASSOCIATED WITH HETERO- THALLISM AND HOMOTHALLISM IN THE ASCOMY- CETE *NEUROSPORA*<sup>1</sup>

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### INTRODUCTION

A new genus of ascomycetes with four species has been recently described by Shear and Dodge (18)<sup>2</sup>. The culture work reported in that paper established the connection between the conidial and the ascocarpic stages. It also proved that two of the species, *Neurospora sitophila* and *Neurospora crassa*, are heterothallic. A preliminary study of the nuclei in spore formation in *Neurospora tetrasperma*, which is ordinarily homothallic, suggested that by a proper selection of spores on the basis of their size, monosporous mycelia could be obtained which would produce only conidia when grown separately, but which when properly mated would also develop ascocarps. The present paper discusses the nuclear behavior which accounts for the fact that heterothallic strains may be segregated out of a species which is commonly homothallic.

The ascus of *Neurospora tetrasperma* usually develops only four spores, and each spore contains two nuclei at its origin. The method by which two or more of the eight nuclei in an ascus cooperate in the delimitation of an ascospore is described for the first time.

### MATERIAL AND METHODS

Material for cytological work on *Neurospora tetrasperma* was obtained from cultures on corn-meal agar in test tubes and Petri dishes. If conidia or fragments of mycelium are sowed on corn-meal agar, perithecia will begin to appear about the fifth day, so that material for fixing may be had from cultures which are from 5 to 10 days old, depending on the temperature of the culture room.

Small pieces of agar bearing ascocarps were fixed in Flemming's weaker solution. Sections were cut 5 microns thick and stained with the triple stain. The perithecia usually form on the surface of the agar, so that orientation of the fruit bodies in cutting is not difficult. Not infrequently the perithecia develop below the surface of the agar. Such material is not as satisfactory for this work. The perithecia of *Neurospora crassa* do not fix as well as do those of *N. sitophila* and *N. tetrasperma*. Only those results obtained by a study of the last species are given in this paper.

<sup>1</sup> Received for publication April 19, 1927; issued October, 1927.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 304.

# NUCLEAR BEHAVIOR IN THE ASCUS OF NEUROSPORA TETRASPERMA

The ascus crosier appears to be formed in the usual fashion and later the two nuclei in the young ascus fuse. In this stage it is usually seen that the cytoplasm of the ascus has been differentiated into two regions. The central part, more finely granular, is to become the spore plasm. The epiplasm in the upper and lower ends of the ascus contains large vacuoles which are somewhat elongated.

After nuclear fusion has taken place and the young ascus has elongated, the development of a thickening at the apex of the ascus goes on until it can be recognized clearly as a red ring when stained with safranin. In fixed material the cytoplasm at both ends of the ascus seems inclined to shrink more than the central portion, perhaps because it is more vacuolar. The fusion nucleus is often surrounded by several so-called extranuclear bodies which stain deeply (pl. 1, A).

The spindle of the first division lies along the long axis of the ascus (pl. 1, B). In no case has a transverse spindle been found at this stage although the ascus is rather broad and there would be sufficient space for a transverse spindle.

After the first division is completed the reorganized daughter nuclei, moving apart, come to rest in various positions. They may be widely separated, one lying in the upper, the other in the lower part of the spore plasm. In that case the spindles of the second division will appear to be nearly longitudinal (pl. 1, D). This material gives an abundance of the two-nucleate stage, but the chromatin does not differentiate well in staining. The chromosomes in the metaphase stages of the first and second divisions are very distinct so that they might be counted without great difficulty. If the daughter nuclei had come to rest in the position shown in Plate 1, C, the two spindles would then be oriented in some such way as are those shown in Plate 1, E and F. At E the remains of the old

## EXPLANATORY LEGEND FOR PLATE 1<sup>1</sup>

A.—Primary nucleus with extranuclear bodies. Cytoplasm differentiated into the central spore plasm, and epiplasm at each end with large vacuoles. Shrinkage during fixation at the upper and lower ends.

B.—Metaphase of the first division. The spindle is parallel to the long axis of the ascus. The pointed ends of the spindle take the safranin stain readily.

C.—One type of the two-nucleate stage where the nuclei are not oriented on the central longitudinal axis of the ascus; large vacuoles in either end of the ascus.

D.—Second division in metaphase stages. The spindles are longitudinal, one somewhat above the other but at a different focus.

E.—Another type of division in which the spindles lie oblique to the walls of the ascus. The remains of the old nuclear membrane somewhat collapsed, show distinctly on both of the spindles. Very little cytoplasm in either end of the ascus; spore plasm rather dense and finely granular.

F.—Late telophase stage of the second division. Spindles oblique, one somewhat above and parallel to the other. The polar chromatin masses connected by filamentous structures which take the stain rather heavily. Remains of the parent nuclear material seen at the center of the upper spindle.

G.—Four-nucleate stage, the pair of nuclei lying at a higher focus, more heavily shaded. Such a distribution of the nuclei may well result from divisions similar to those shown in Figures E and F. The two upper nuclei are somewhat pear shaped. The relationship of the nuclei can be determined by the location of the central bodies; compare with I.

H.—Two views of the same nucleus somewhat more enlarged than in the other figures. At the left and seen at a higher focus, the nucleus appears nearly spherical or without particular distortion. At the right, part of the same nucleus, seen at a lower focus, now shows the beak-like cap with forked appendages.

I.—Four-nucleate stage in resting condition. Nucleoles distinct. Each nucleus is pear shaped or crowned by a beak-like cap of fibers at the end of which is a forked appendage. The beaks of the sister nuclei extend in opposite directions. The spore plasm at this stage is divided into two parts by a series of central vacuoles.

J and K.—Two sections of the same ascus during metaphase stages of the third division. J should have been mounted so that the nucleus would have been at about the same level as the lower nucleus in K. At the upper end of the ascus in K can be seen one complete spindle and the tip end of a second, the remainder of which appeared in the next section and was not drawn.

<sup>1</sup> The writer is indebted to Lulu O. Gaiser for assistance in drawing a number of the figures included in the plates.

nuclear membranes and the fibrous material connecting the sister polar masses are still visible. Slightly later stages are shown in F. The daughter nuclei in each pair are beginning to reorganize while the darkly staining connecting strands are still very prominent.

In about half of the figures of the second division catalogued the spindles present the appearance of conjugate division. They lie more or less parallel to each other and oblique to the long axis of the ascus. Divisions in the ascus have heretofore been regarded merely as simultaneous. Conjugate divisions are devices to insure nonsister nuclei being brought together in a common cytoplasm. With such a method of division as is shown in Plate 1, E and F, each end of the ascus would surely contain a pair of nonsister nuclei. If such an arrangement serves a useful purpose, and it would if segregation of the sex factors occurs in the first division, how can such a contingency be provided for in case the dividing nuclei are located as shown in Plate 1, D? Fully 50 per cent of the division stages at this time actually show these spindles rather widely separated. This would suggest that after the second division there would of necessity be a pair of sister nuclei in each end of the spore plasm all in one row. The writer has never seen such an orientation in *Neurospora tetrasperma*. Since they do not lie on the same axis of the ascus, a shifting of position of the reorganizing nuclei, still connected by fibers, must take place, so that a pair of nonsister nuclei will come to lie in each end of the ascus. This is similar to the shifting of pairs of nuclei in the promycelium described by Dodge and Gaiser (5) for *Caeoma nitens*, except in a reverse order and with the opposite effect.

It will be shown later in the discussion that perhaps nothing in the cytology of the ascus is more important in its bearing on the experimental results obtained from growing ascospores and crossing different species of *Neurospora*, than is a knowledge of the genetic relationship of the two nuclei which one sees in each end of the ascus after the second division. Practically any section of a perithecium in which the asci are developing vigorously will show such figures as are given in Plate 1, I. Because of the beautiful symmetry presented in such cases there can be no doubt that the nuclei whose beaks extend in opposite directions are sister nuclei.

Each of the four nuclei shown in Plate 1, G, is slightly elongated. A central body is clearly evident at the point of greatest elongation of each nucleus—that is, on the side toward the top of the figure for the two upper nuclei and on the side toward the bottom of the figure for the two lower ones. The nuclei that are more heavily shaded

#### EXPLANATORY LEGEND FOR PLATE 2

A to C.—Three consecutive sections of the same ascus. The first section, A, shows a large part of one spindle and a part of one spike-like appendage of another. The rest of these nuclei appear at the lower end of the ascus, shown in B. The chromatin material is bunched at each end of the spindles and spike-like prolongations somewhat curved, extend out to the plasm membrane. The two nuclei shown in the upper end of the ascus, B and C, are members of a pair dividing conjugately, one almost directly under the other but more obliquely placed.

D.—Slightly older or late telophase stage. The daughter nuclei nearly reorganized and showing the curved spiked extensions now pressing against the plasma membrane on each side.

E and F.—Two adjacent sections of the same ascus showing the eight nuclei at the beginning of spore formation. The nuclei clearly in pairs, symmetrically placed, two on one side and two on the other in each end of the ascus. The appendages are now strongly curved and from these structures are proceeding very delicate astral rays. The spore plasm of the ascus is divided into two regions by large vacuoles arranged across the ascus at about the middle.

G.—Stage in spore formation in one end of an ascus somewhat later than the stages shown in E and F. Each spore will contain a pair of nonsister nuclei from the beginning.

were at a higher focus. They are undoubtedly sister nuclei. The best evidence for this assumption is that the central bodies are symmetrically located on opposite sides of the nuclei. Viewed at right angles to the plane of the section shown in Plate 1, G, such a disposition of the four nuclei could well have followed from an orientation of the spindles like that shown in E.

The writer has described the nuclei at this stage as pear shaped or as having long beaks (pl. 1, I). When, however, one studies such sections as are illustrated in Plate 1, H, or those of more delicately stained preparations, the beak sometimes appears to be made up of a cone of fine fibers capping the nucleus, which perhaps is only slightly elongated. In spite of this evidence the writer is not convinced that such figures represent early stages in the reorganizing of the daughter nuclei.

Another reason why Plate 1, I, is assumed to represent a stage long after the second division, is that the spore plasm is now very definitely divided into two sections by a series of vacuoles extending somewhat obliquely across the ascus. These vacuoles enlarge and run together more and more after the third division (pl. 2, E and F.) The organization of the fine granular material at the poles (pl. 1, E) suggests slightly the origin of the forked appendages, yet at the poles of the spindle in F there is not the slightest indication of such structures. Plate 1, F, clearly represents a later stage than E.

A study of slightly later and more critical stages may show that the antlerlike structures, central bodies(?), attached to the chromosomes by fibres, simply break in two and move straight out to the walls of the ascus on opposite sides, giving metaphase stages like those shown in Plate 1, J and K.

In the third division the spindles are transverse—that is, perpendicular to the ascus walls. In the metaphases of all three divisions one sees at each end of the spindles a sharply pointed mass of deeply staining substances, a part of which is undoubtedly the centrosome, although astral rays do not show at all prominently at any stage in this material.

Usually not all of the four spindles of the third division lie in one plane. The section may show a longitudinal view of the upper spindle, while the lower spindle appears only in a polar view. The sections for the illustrations for Plate 1, J and K, and Plate 2, A and C, were chosen particularly because, showing longitudinal and not polar views of the spindles, they give the best idea of the relationship of the eight nuclei resulting from this set of divisions.

In the telophase stages the now curved and more densely staining end structures appear to be attached to the ascus walls, the chromatin bodies being bunched together in two or three masses (pl. 2, A, B, and C) and the remains of the old spindle still stretching across the ascus. A still later stage is shown in Plate 2, D, in which the reorganization of the daughter nuclei is about completed.

The next stage shows pairs of small nuclei side by side, pressing against the ascus wall. Symmetrically placed on the opposite side of the ascus, is another pair of similar nuclei (pl. 2, E and F). Seldom are all four nuclei seen in one section. Serial sections, however, invariably show the nuclei which were missed in the preceding section.

The beaks of the nuclei now become further prolonged and curved sharply at the ends like umbrella handles. Astral rays are very

delicate in these species so that the details in spore delimitation are not adequately brought out. There can be no doubt, however, as to the nature of the process or of the fact that adjacent nonsister nuclei cooperate in cutting out the spores. In the early stages it is sometimes impossible to tell whether one very large spore or two of normal size will be delimited in one end of an ascus, especially in cases where four nuclei lie in nearly the same plane (pl. 2, G). If the plasma membrane and vacuoles take part in the process, it is only in a secondary way. Large vacuoles often appear both within and outside the spore opposite the nuclei (pl. 3, A and B).

There must be much twisting, turning, and slipping of the spores just as soon as they are fully cut out; otherwise they would not be uniseriate at maturity, and such sections as are shown in Plate 3, A, in which the two nuclei in each of the four spores all point in the same direction, would not be found. Three such cases were observed in this material. The nuclei in adjacent spores usually extend in opposite directions (pl. 3, B, above). The curved tip ends of the beaks must be fairly definite structures (pl. 3, C). With delimitation complete, the body of the nucleus becomes detached from the curved end (pl. 3, D) and moves down to the center of the spore (pl. 3, E). After the four spores have enlarged somewhat and become more or less elliptical, their nuclei divide simultaneously. The spindles in a spore usually are not in the same plane. Parts of two adjacent sections of the same ascus are shown in Plate 3, F and G. The spindles of the spore at *a* and *a'* are at right angles. The mature normal spore will have four nuclei (pl. 3, H).

#### SIGNIFICANCE OF ABNORMAL SPORES

The most interesting phase of spore formation is brought out in Plate 3, B, where the formation of four spores, each with a single nucleus, is going on in one end of an ascus while two spores with two nuclei each are being cut out in the other end. The genetic relationship of the nuclei in the uninucleate spores can be told by the position of the beaks, which extend in opposite directions when the nuclei are sisters. The two adjacent spores at the right of Plate 3, B, as well as the two at the left would have been of opposite sex; but it is problematical where they would finally lie in the mature ascus. In a narrow ascus, such as is found in *Neurospora crassa* and *N. sitophila*, the two spores developed from sister nuclei are always adjacent in the mature ascus. Further work is necessary to learn the details of the processes by which more than two nuclei cooperate in spore formation in *N. tetrasperma*. As noted previously, an ascus of this species normally contains four spores (fig. 1, A). If one crushes four or five perithecia in which asci are maturing, he will probably find one or two asci with an abnormal number of spores (fig. 1, B to E); most rarely of all, an ascus may contain only one giant spore. If the nuclei of one sex be indicated in black and those of the other sex in white, Figure 1, F and G, will represent the way in which the nuclei cooperate in cutting out spores with two nuclei, and also the way in which they sometimes fail to cooperate, with the result that small uninucleate, unisexual spores are developed. Figure 1, H and I, show how either four or eight nuclei could act together in cutting out large spores. This point has not been followed up.

Shear and Dodge (18) found that monosporous mycelia from small spores develop large numbers of sclerotia or bodies which resemble aborted perithecia. No ascospores are developed in such structures. By properly mating these cultures, normal perithecia are formed with asci which have commonly only four spores. The larger, normal spores, have two nuclei when first delimited and thus contain nuclei of both sexes.

These conditions are in an entirely different category from those described by Burger (3). *Cunninghamella* is heterothallic. Burger reports isolation of monosporous mycelia which he says may be called hermaphroditic. Work with these aberrant mycelia was entirely discontinued so that the cultures were lost and this work has not been repeated or confirmed. What little is known of the cytology of the Mucoraceae would suggest that Burger may well have been correct in his conclusions. The condition described might depend on where and how completely segregation takes place, and the chances for the accidental inclusion of nuclei of both sexes in the same spore at some critical point following the germination of the zygospor. Such an explanation, however, is discounted by Burger, who is inclined to entertain the idea of compatibility, although he has no explanation to offer for the condition which he describes.

The writer refers to monosporous mycelia of the heterothallic species as haplonts and uses the terms "unisexual," "bisexual," "sex," and "sexuality" in this paper without knowing, in fact, whether or not the mycelia of the species of *Neurospora* produce functional oogonia and antheridia. Both reciprocal haplonts produce coiled structures which enter into the development of the perithecium. Further study will no doubt demonstrate that these primordia are morphologically and functionally different. Work on crossing of the haplonts of different species, to be reported later, shows that the descendants of nuclei from both haplonts must finally fuse in the ascus.

Knowing that the existence of "psuedo-heterothallism" in *Neurospora tetrasperma* is based on the accidental inclusion of only one of the two sexually different nuclei which are normally contained in each spore at its origin, a way is suggested by which one may be able to obtain homothallic strains from heterothallic species, such as *N. crassa* and *N. sitophila*. The former species particularly may,

#### EXPLANATORY LEGEND FOR PLATE 3

A.—Section of an ascus showing four spores just after they have been completely delimited. Each spore contains a pair of nuclei close together. In this case the spores have readjusted their positions so the beaks in the four pairs all extend in the same general direction. The epiplasm is considerably shrunken from fixation and the spores have not as yet assumed their final elliptical shape. Very fine astral rays are still visible proceeding from the curved projections of the nuclei.

B.—Ascus showing the basis for the development of homothallic and heterothallic spores in the same ascus. Each of the two spores in the upper end of the ascus contains two nuclei of opposite sex. Each of the four spores in the lower end of the ascus contains only a single nucleus. Since the nuclear beaks of the two spores at the right extend in the same direction, they are probably of opposite sexes. Each spore of the pair at the left is also unisexual. The spores with one nucleus are much smaller than those containing two.

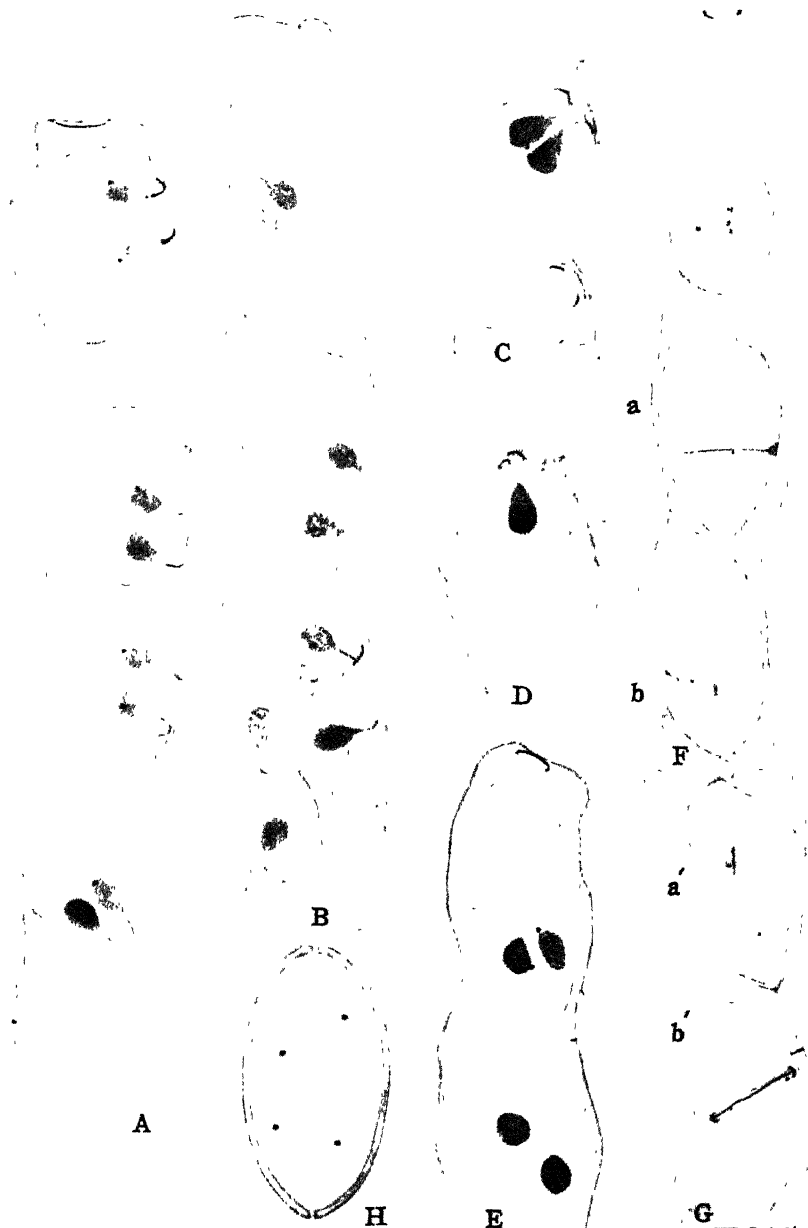
C.—Later stage in spore formation. The nuclei about to break away from the tip ends of the curved beaks. In the lower of the two spores, the appendages which were cut off in sectioning are indicated at the right.

D.—Still later stage in spore formation. The nuclei have been completely detached from the appendages, which are still visible at the upper end of the spore.

E.—The daughter nuclei have become nearly rounded up and the spores no longer show the remains of the hooked nuclear beaks.

F and G.—Parts of two sections of the same ascus showing dividing nuclei in telophase stage. At a and a' are shown two sections of the same spore, and at b and b' two sections of the next lower spore. Two nuclei in each of the four spores of this ascus were all in the same stage of division.

H.—A mature spore after reorganization of the four daughter nuclei and the thickening of the spore wall; a germ pore is visible at each end.



(For explanatory legend, see p. 294)



on rare occasions, develop an ascospore which is much too large for a spore with only one nucleus at its origin (fig. 1) and can not be accounted for as simply a matter of competition for the nourishment in the spore plasm. Should such a spore contain two or more nuclei at its origin (fig. 1, G, H) and have been delimited in the same way as are the normal spores of *N. tetrasperma*, then, on germination, a homothallic mycelium will be developed. Clearly heterothallism and homothallism in the species of *Neurospora* are not absolutely fixed specific characters, although the sexual nature of an individual haplont is definitely determined by the time the spore is cut out.

The occurrence of spores of different sizes in the ascus of *Bulgaria inquinans* has long been a matter for speculation. Moreau (14) finds that some spores are large and others are small for two very different reasons. All of the nuclei do not necessarily divide simultaneously so that one may sometimes find resting nuclei of both the second and third generations in the ascus at the same time. A nucleus

of the second generation will be much larger than one of the third. Spores formed at once and including nuclei of the third division, this author believes, having the first chance at the nourishment in the spore plasm, would become larger than the other spores formed in the same ascus later. Regardless of the size of the spore, each one contains only one nucleus at its origin. When two of the original eight nuclei are included in the same spore, as Wolf (20) found in *Podospora anserina*, the spore will be much larger than one with only a single nucleus. Such forms as *P. anserina* and *Bulgaria polymorpha* should prove extremely interesting. Has the former a heterothallic sister species? May not the smaller uninucleate spores of the latter be unisexual and the larger binucleate spores be bisexual as they are in *Neurospora tetrasperma*? Faull (6) figures a two nucleate spore of *Neotiella*, and Fraser (9) shows a spore of *Humaria rutilans* containing five nuclei at its origin. These authors considered such spores simply as abnormal and of no consequence. The writer has pointed out above the significance of the inclusion of different numbers of nuclei in spores, particularly in cases where heterothallism must be taken into account.

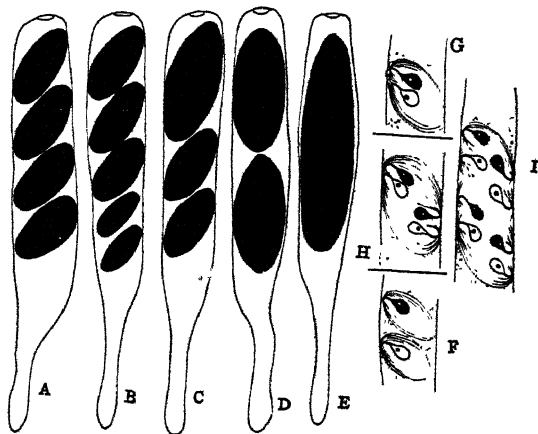


FIG. 1.—Diagram of ascus with various numbers of spores at maturity and method of spore delimitation. A, normal number of spores is four, very rarely only one giant spore (E) is formed; F, small uninucleate, unisexual spores; G, bisexual, binucleate spores; H, bisexual four-nucleate spore; I, eight-nucleate spore in which all of the nuclei resulting from the third division of the fusion nucleus cooperate in cutting out the spore. H and I theoretical

#### HETEROTHALLISM NOT DIOECISM

Perhaps 50 per cent of the red algae are heterothallic. In the filamentous and thalloid green and brown algae the male and female

gametes of certain species are borne on different plants. The same is true for liverworts, mosses, and ferns; and, of course, all seed plants are heterothallic. Among the fungi, certain species of water molds have long been known to be heterothallic. Thanks particularly to Thaxter, one order of ascomycetes alone, the Laboulbeniales, now presents at least a dozen genera with some hundred species which are heterothallic. In all of the groups mentioned, it is readily demonstrated that some gametophytes are male and others are female, although the cytological details of nuclear behavior may be entirely unknown. With such evidence at hand, it is strange that the existence of heterothallism among our higher ascomycetes should have been so long unknown.

Blakeslee (2), when he first proposed the use of the terms, stated that "heterothallic" and "homothallic" correspond to "dioecious" and "monoecious" in the flowering plants. In all his subsequent writings this author shows that originally he must have intended the illustration merely as an analogy. Allen (1) working on the genetics of *Sphaerocarpos* has emphasized the importance of keeping in mind the difference between male and female plants of the liverworts, mosses, etc., on the one hand, and staminate and pistillate individuals of the flowering plants on the other. Pollen tubes are male haplonts, embryo sacs are female haplonts. Recently Sharp (17) in discussing the factorial interpretation of sex determination rightly insists that all flowering plants are heterothallic. Only a comparatively small number are dioecious. Less confusion might result if, for example, such genera as *Amorphomyces* and *Herpomyces* were referred to as heterothallic instead of as dioecious. The terms now have entirely different meanings.

The Mucoraceae apparently do not afford the best material for cytological study. No one has been able so far to find any connection between nuclear behavior and the segregation of the factors determining sexual reproduction in heterothallic forms.

In the case of certain higher basidiomycetes it is now known just what the nuclei do, step by step, from the time the spores germinate until the new generation of spores is formed on the basidium. No more complete story of nuclear behavior is known in the plant kingdom, but there is nothing in the cytology which serves as a basis for predicting which species of *Coprinus*, for example, will be heterothallic and which ones homothallic.

*Melanospora destruens* (18) resembles *Neurospora sitophila* in several morphological features. It is not yet clear why the former should be homothallic and the latter heterothallic. Within the genus *Neurospora*, however, the factors which determine certain types of sexual reaction are clearly segregated before, instead of after, spore formation, so that a study of nuclear behavior in the ascus enables one to see why one species is heterothallic while another is homothallic.

#### SEGREGATION OF SEX FACTORS

In *Pyronema* the antheridia are developed on dichotomously branched hyphae which are quite distinct from those that build the oogonia. Monosporous mycelia of this species are said (4) to produce ascocarps in culture. Each ascospore is at first uninucleate. The segregation of the sexes must therefore take place a long time after

spore germination. A number of ascomycetes outside of the Laboulbeniales develop sex organs which are morphologically and functionally of two different sorts. Since the two spores in an ascus of *Phyllactinia corylea* are formed from sister nuclei (11) the chances, one might think, for this species to be heterothallic are small. Yet if the factors for sex differentiation are segregated out in the third division, as may well be the case, spores of two different sexes will then be formed in the same ascus. The production of perithecia by certain species of the Erysiphaceae is very erratic. When culture work on such forms is carried out with monosporous haplonts, no doubt some of these species, such as the mildew on raspberries, will likely be found to be heterothallic.

Thaxter's highly interesting accounts of the development of various species of the Laboulbeniales show that in the heterothallic ("dioecious") forms the spores are of two sorts, usually somewhat different in size. The male spore is frequently somewhat smaller than the female. They are discharged from the asci in pairs and become attached to the host side by side. The sex of each spore has been determined absolutely by the time it has been delimited. In this connection Thaxter remarks (19, v. 13, p. 222): "The cytological phenomena \* \* \* which lead up to the sexual differentiation and pairing in the ascus of these male and female spores, is likely to prove of unusual interest." It is a curious fact that in cases where the spores of the hermaphroditic species are frequently discharged in pairs and become attached to the host side by side, only one of the spores of the pair develops into a mature plant (19, v. 14). The other spore degenerates without germination.

Thaxter (19, v. 12, p. 217-218) further says: "It has been previously mentioned that in a majority of forms [hermaphroditic species] the antheridial appendage is developed from the terminal cell of the germinating spore. The female organs, however, are always formed from the products of the division of the basal cell, never in any case from the terminal cell, where this is present. Although the products of the division of the terminal cell are invariably sterile or male, it is not true, as might be supposed, that the basal cell or its derivatives have any inherent female character, since in many cases both normal and abnormal antheridia and antheridial branches may arise below the point of insertion of the female organ, or even, in exceptional cases, replace it entirely."

This may be the correct interpretation. It may also be claimed that the facts cited go to show conclusively that usually the terminal cell is in fact inherently male and the basal cell female, and that segregation of the sexes takes place during the fourth nuclear division after nuclear fusion, that is, during the first nuclear division after spores have been cut out. Occasionally, however, such a sex segregation is not consummated at this time and antheridial appendages develop from the cell which, normally or abnormally, gives rise to the carpogenic structures. This could very well be due to a failure to lay down the septum until after a second nuclear division in the spore, and the inclusion of one of the extra male nuclei in the basal cell. If the development of the male structures from the terminal cell and the female structures from the basal cell could be reversed

by inverting the spore or in any other way, this would prove, of course, that there is nothing inherent sexually in either segment of the spore.

Thaxter has been unable to determine whether or not the asci of the genus *Herpomyces* are eight-spored. "If this were actually the case," he says, "it would involve the curious phenomenon of absolute sex differentiation in the last mitosis, which would not necessarily occur in a four-spored ascus" (19, v. 13, p. 222).

It is to be regretted that the only cytological work so far published on the *Laboulbeniales* has been upon two species in which the antheridial structures are said to be unknown, so that sexual reproduction as such would clearly be out of the question. Faull (8) finds that in *Laboulbenia chaetophora* the spindles of the first and second mitoses are longitudinal. In the four-nucleate stage the nuclei are in pairs, two in the upper part of the ascus and two in the lower; again in the third division the spindles are longitudinal. After reorganization, four nuclei move to the upper part of the ascus and disintegrate. The four which develop at the lower ends of the spindles take a central position. Faull says further (?): "The functional nuclei, in *Laboulbenia chaetophora* at least, are the lower ones in the spindles of the last mitosis. There are some reasons for believing that the same is true of *Amorphomyces* and *Dioichomyces*, in which case sexual differentiation of the spores might be determined in the second division."

Assuming that nuclear behavior during the processes leading up to the delimitation of the ascospores should be the same for the heterothallic genera as that given by Faull for the two parthenogenetic species which he studied, is it necessary to assume also that the segregation of the sex factors must occur in the second division? For practically all the species of this group where fecundation is an essential factor for the full development of the ascus, all that would be necessary to make a species heterothallic would be to move the point of segregation back from the fourth to the third nuclear division. Evidently many of the species commonly homothallic have provided for just such an emergency by discharging the spores in pairs.

The features in the cytology of the ascus discussed by previous authors and which have a particular bearing on segregation of the sex factors and other work covered in this paper are: (1) The position and direction taken by the spindles during each of the three nuclear divisions; (2) the location of the resting nuclei in the spore plasma and their relationship, one to the other, at different stages in the development of the ascus; and (3) the part taken by the nuclei during spore formation and the method of spore delimitation. It may be noted that on certain points there is a general agreement among previous writers.

The spindle in the first division of the primary nucleus usually lies parallel to the long axis of the ascus. Harper (10) and Clausen (4) have pointed out that in *Pyronema* this spindle may also be transverse. The writer has found that in *Ascobolus winteri* the first spindle is always transverse. One or two other exceptions have been reported.

After the first division has been completed, the two daughter nuclei separate and come to lie one above the other, either on the

median long axis of the ascus, or, just as frequently, on some line which is oblique to the ascus walls. Reports of finding the nuclei lying side by side as though reorganized at the ends of a transverse spindle have not been seen in the literature.

The two spindles of the second division are usually described as lying along the general direction of the ascus axis. In *Pyronema*, according to Claussen (4), and in *Phyllactinia*, according to Harper (11), one of the two spindles of the second division may be transverse. As a general rule when an ascus is long and narrow the four nuclei, following a second division, lie in a row on the long axis of the ascus. The two nuclei in each end of the axis of the ascus would then be sister nuclei. The disposition of the four nuclei must depend largely on how much of the cytoplasm of the axis is differentiated into spore plasm. Jolivette (16) says that if a transverse section of the ascus were made after the second division in *Geoglossum glabrum*, the four nuclei would be seen in the same plane. In a longitudinal section two nuclei are seen in one focus and two in the other. The spindles of the third division show no tendency to lie in the transverse axis of the asci.

There are a few other reports of the lack of uniformity in the disposition of the spindles in the third division. Faull (7, 8) claims that all four of the spindles of the third division in *Laboulbenia* are longitudinal. In most other ascomycetes, however, they are reported to be more or less transverse. Komarnitzky (12) and Faull have both remarked on the possible connection or bearing which the genetic relationship of particular nuclei may have on "dioecism" in the *Laboulbeniaceae*. Their work will be considered further in the general discussion. In most ascomycetes division figures are so rarely found that one should not draw conclusions as to the significance of the orientation of the spindles in certain cases in which it is clear that the author has based his report on only one or two spindles which he was fortunate enough to find.

The first spindle in *Verpa bohemica* (12) is nearly longitudinal. The spore plasm is developed in the upper end of the ascus and all of the nuclear divisions occur in this end. Of the two spindles in the second division, the upper appears to be transverse, the lower somewhat oblique. The only spindle shown in the third division is nearly transversely placed. The evidence presented by Komarnitzky's figures is not conclusive as to the particular nuclei which are concerned in spore formation. Two spores are finally cut out and the other six nuclei degenerate. Komarnitzky realizes, however, the importance of knowing the exact orientation of the spindles during the three different divisions in the ascus.

If no switching or exchange of position of the nuclei takes place after their reconstruction, in other words, if one is justified in drawing conclusions as to the genetic relationship of particular nuclei from the position and orientation of the division figures, then in the "dioecious" forms of the *Laboulbeniaceae*, segregation of the sex factors may very well take place in any one of the three divisions with the same result, provided the nuclei take the positions and the spindles are oriented as Faull (8) figures for *Laboulbenia*. In all three divisions the spindles extend lengthwise of the ascus. Faull (8, fig. 57) shows that the four nuclei resulting from the second mitosis lie in pairs, two in one end of the ascus and two in the other, comparable

to what occurs in *Neurospora tetrasperma* (pl. 1, I). It will appear in due time, however, that one would be led far astray were he to presume to draw conclusions as to the time of segregation and the sexual nature of the eight spores in the heterothallic species *N. sitophila* from what he finds to be true of nuclear behavior in the homothallic form *N. tetrasperma*.

Full information regarding the early prophase stages as the four nuclei of *Neurospora tetrasperma* go into the third division, has not been obtained. This is a critical stage, not only because of its bearing on the question as to where segregation takes place, but also because further light is desirable as to the nature of the structures which always appear as forked appendages at the beaks of the nuclei at this time. If these horns represent merely lines of flow, or a massing due to fixation of astral rays, one would not expect to find such regularity in their form and position. They certainly appear more like definite organs, in the nature of monstrous central bodies or blepharoplasts.

Harper (11) shows that immediately after the first division the two nuclei in *Phyllactinia* are pear-shaped. He states that they always round up, however, before beginning the second division. The same thing may take place in the case of the four nucleate stage of *Neurospora* so that the stage shown in Plate 1, I, would precede that shown in Plate 1, G. There is much evidence against such a view, however. First, the two pairs of nuclei undergoing the third division are at opposite ends of the ascus and widely separated. There is practically no change of position after the hornlike appendages have developed. In the stage shown in Plate 1, G, the nuclei are not in the final position for the third division. The beaked nuclei in Plate 1, I, may not be fully mature or reorganized, yet they are certainly in a resting condition and must so endure for a long time. Such stages are very abundant in the material. If the nuclei dispose of the beaks and forked appendages before going into the third division, they must do so in a very short space of time. With the development of the spindle of the last division, each end of it is provided again with a curved spikelike structure which extends out to the plasma membrane. After the first division on the contrary the two nuclei round up and remain in this condition for some time.

Since the factors upon which sexual reproduction in the heterothallic species of *Neurospora* depends are lodged in two different spores and in two different nuclei of the same spore in homothallic species, it is essential to a proper understanding of the questions involved to determine at what time and in what way the segregation of the sex factors takes place.

Shear and Dodge (18) have reported that in *Neurospora crassa* four spores in an ascus are of one sex and four of the other. Nothing is said concerning the position in the ascus of each of the eight spores.

Students of heterothallism of the basidiomycetes, forced to explain some of the curious results of their culture work, are assuming that two pairs of factors, "sex factors," control the reactions leading to the formation of clamp connections. Newton (15) believes that in *Coprinus lagopus* segregation of one factor may take place in the

first division in the basidium and the segregation of the other factor in the second division. The writer has not been confronted with such complicated situations so far, yet the results obtained by crossing *Neurospora sitophila* with *N. tetrasperma* show the need of exact knowledge of nuclear behavior through the entire life cycle in both species as well as in the different hybrid generations. It will be shown in the following diagrams that nuclear behavior in the ascus of *N. tetrasperma* is such that normally totipotent or homothallic ascospores will be formed, regardless of whether segregation takes place in the first, second, or third division.

One may take as a type an ascus such as that of *Galactinia succosa* figured by Maire (13), which shows very diagrammatically the position of the nuclei and orientation of the spindles generally reported for long asci. If segregation of the sex factors takes place in the first division, then as indicated in the diagram (fig. 2) the four spores in the upper end of the ascus must be all alike, and all of one sex, and the four in the lower end must be of the other sex. If segregation of the sex factors takes place in the second division in a species the spores will alternate in pairs, the first two being of one sex and the next two of the opposite sex. With a knowledge of conditions in the ascus of *N. tetrasperma*, a brief study of nuclear behavior in the ascus of *N. sitophila* has convinced the writer that the spores of the latter species will so alternate in pairs. Should segregation take place in the third division, then the four spores in one end of the ascus will alternate as regards their sex. So it is clear that with no shifting of position of the nuclei, three different pictures will result, depending on the place of segregation.

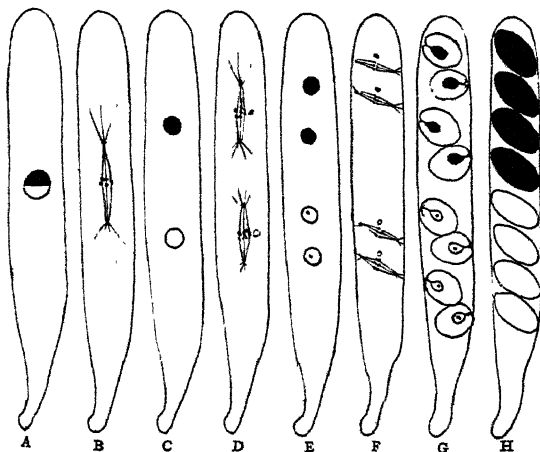


FIG. 2.—Diagram showing nuclear behavior and position of the four spores of each sex in a typical long ascus if segregation of the sex factors should occur in the first mitosis

In *Neurospora crassa* and *N. sitophila*, after the second divisions, the four nuclei lie in a row in the ascus. In no case has the writer found, as previously noted, such a condition in *N. tetrasperma*. The evidence so far discovered indicates that regardless of the position of the two nuclei during the second division in the last species such a shifting must take place as is necessary to bring the four nuclei into the formation shown in Figure 3, E, which is the one picture that seems to be very constant in this material (pl. 1, I).

Should segregation take place in the first mitosis in *Neurospora tetrasperma* the way in which each spore would receive at its origin one nucleus of each sex is indicated in Figure 3, A to H. The spindle of the first division is always longitudinal. The essential difference

Should segregation take place in the first mitosis in *Neurospora tetrasperma* the way in which each spore would receive at its origin one nucleus of each sex is indicated in Figure 3, A to H. The spindle of the first division is always longitudinal. The essential difference

between this type of nuclear behavior and that shown in Figure 2 is not in the number of nuclei in the young spore. In a number of ascomycetes with long asci it has been demonstrated, as can be done

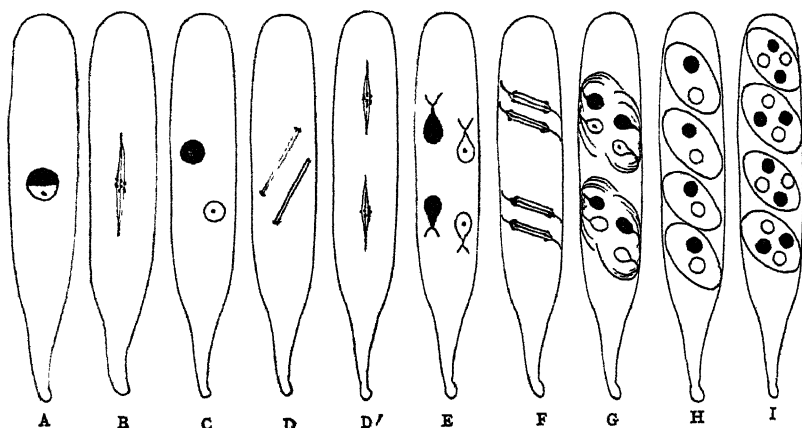


FIG. 3.—Diagram showing nuclear behavior and method of formation of bisexual spores in *Neurospora tetrasperma*. The position of the spindles in the second mitosis may be parallel to each other and oblique to the long axis of the ascus, D, or they may be longitudinal, one in each end of the ascus, D'.

easily in case of *N. sitophila* and *N. crassa*, that the two nuclei in each end of the ascus are sisters (fig. 2, E). With segregation taking place in either the second or third division such an arrangement would accord with the experimental results obtained with *N. tetrasperma* should two adjacent nuclei be included in the same spore. Yet the evidence obtained by a study of the shape and the position of the four nuclei of this species after they have come to rest (fig. 3, E) indicates that here, as previously noted, a shifting of the nuclei must occur after divisions like those shown in Plate 2, D, or Figure 3, D'; otherwise fully 50 per cent of the asci at this stage would show four nuclei in a row down the center, which certainly is not the case. No such orientation has been found, although this would account for the occasional formation of large spores which are unisexual.

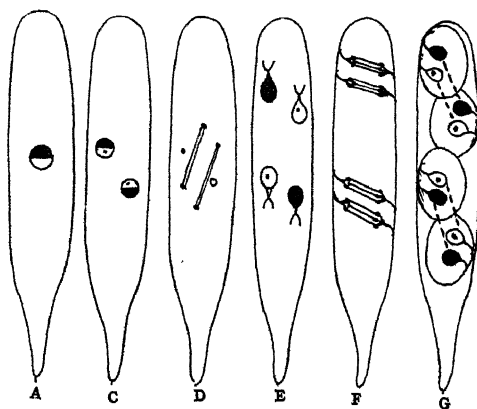


FIG. 4.—Diagram of nuclear condition leading to the development of bisexual spores in *Neurospora tetrasperma*, should segregation of the sex factors occur in the second mitosis, other conditions as in Figure 3.

With segregation in the second division in *Neurospora tetrasperma*, the spores will all be bisexual (fig. 4, A to G) no matter in which of the two ways (fig. 3, D or fig. 3, D') the spindles of the second mitosis are oriented. Occasionally in the case of this species, only one

nucleus is included in each of certain spores (pl. 3, B). With nuclear behavior otherwise the same, such spores would alternate in pairs in the ascus, two being of one sex and two of the other.

Figure 5, A to G, shows how segregation might take place in the third division without preventing the formation of bisexual spores. Perhaps 1 per cent of the monosporous mycelia from what was judged to be either normal spores, or spores which were oversized, failed to develop perithecia. One is forced to find an explanation for this apparent failure in such cases to include nuclei of both sexes in these spores. A section of an ascus which was taken to be abnormal showed what appears to be a pair of nuclei in the second division, with spindles transverse, one in the upper, the other in the lower end of the ascus. Such abnormal conditions are generally said to be of no particular significance, yet these cases may be the very ones to account for unusual conditions, such as the development of large unisexual spores with two or even more nuclei at their origin.

The sexual nature of each of the eight spores in the ascus of *Neurospora sitophila* has been determined culturally by Marguerite Wilcox who has assisted the writer in the preparation of slides for this cytological study. Her report on this work, together with a further account of the cytology of the ascus in this species, is forthcoming. With the cytology of the ascus known and the sexual nature of each of the spores in the ascus of *N. tetrasperma* and *N. sitophila* worked out culturally, the way is cleared for a better interpretation of the results being obtained by crossing reciprocally unisexual haplonts of these two species.

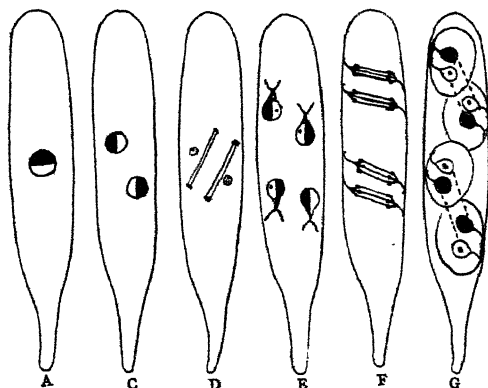


FIG. 5.—Diagram of nuclear condition in an ascus of *Neurospora tetrasperma*, showing the possibility of the formation of bisexual spores, should segregation of the sex factors occur in the third mitosis, other conditions as in Figure 3

#### SUMMARY

*Neurospora tetrasperma*, which is normally homothallic, develops asci with four bisexual spores, as contrasted with the heterothallic species, *N. sitophila*, in which the asci are eight spored, and each spore is unisexual. The cytological basis for this difference is reported.

In *Neurospora tetrasperma*, the spindle of the first mitosis is longitudinal; the two daughter nuclei separate and come to rest one somewhat above the other in the ascus.

With respect to the position and orientation of the spindles of the second division two types are described. In the first type the spindles may lie nearly parallel near the center of the ascus, usually somewhat oblique to the long axis, suggesting conjugate division. In the second type, the spindles are longitudinal, one in each end of the ascus.

Each of the four nuclei is pear shaped and the beak is capped by a forked appendage. The nuclei are arranged symmetrically, two nonsister nuclei in each end of the ascus.

The spindles of the third mitosis are nearly transverse. Normally two adjacent nonsister nuclei cooperate in the delimitation of each ascospore through the development of astral rays from their curved beaks. It is shown how, regardless of whether segregation takes place in the first, second, or third division, each spore will contain one nucleus of each sex.

Occasionally adjacent nuclei, for some reason, are not entirely compatible and fail to cooperate in the cutting out of a spore. Each nucleus then acts independently, and as a result uninucleate, unisexual spores, which are comparatively small, are delimited. Very rarely all eight of the nuclei in an ascus cooperate in the delimitation of a spore. Such an ascus will contain only a single giant spore at maturity.

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# THE TWO MOST COMMON DECAYS OF COTTON BOLLS IN THE SOUTHWESTERN STATES<sup>1</sup>

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## INTRODUCTION

It has been frequently reported from cotton-growing sections of the Southwest that immature cotton bolls are subject to a peculiar form of disease to which the name "smut" has been commonly applied by the growers. The writer has made a survey of cotton fields in California and Arizona and in the section of Mexico immediately adjoining the Imperial Valley and has obtained a considerable number of cultures from affected specimens. These cultures, as well as subsequent inoculation tests, both in the field and in the laboratory, show very clearly that the disease in question is not a smut. Although it is true that in its final phase of development when the boll has dried up it bears a slight resemblance to smut, its initial stages are plainly those of a soft decay.

This soft-decay form either was overlooked by the average observer or, if noticed, was not correlated with subsequent and more pronounced symptoms. Disease symptoms appear limited to bolls that have previously suffered injury by insects or otherwise.

The writer's studies have also revealed the fact that in reality there occur in the field two forms of the boll decay, both of which generally have been known as "smut." One of these is caused by *Aspergillus niger* Van Tiegh. and the other by *Rhizopus nigricans* Ehr. Although on close examination these forms show very distinct peculiarities throughout the entire cycles of their development, they have nevertheless a certain similarity in their general appearance during the fruiting stages of the causal organism. This similarity undoubtedly is responsible for the confusion of the two troubles, particularly since the decay symptoms formerly had not been recognized.

## ASPERGILLUS NIGER

Although very common on all kinds of decaying vegetable matter, the fungus *Aspergillus niger* has heretofore been associated by investigators with only a few diseases of economic plants. Van Pelt (10)<sup>3</sup> considered it to be responsible for a serious black mold of onions in Ohio. Certain laboratory experiments carried on by the writer (7) revealed it to be among important potential parasites of the potato tuber. The same fungus has been definitely shown by Phillips and Smith (6) to be the cause of the so-called smut of white fig varieties in California.

<sup>1</sup> Received for publication April 18, 1927; issued October, 1927.

<sup>2</sup> The writer expresses appreciation to W. B. Camp, of the Office of Cotton, Rubber, and other Tropical Plants, Bureau of Plant Industry, for the great amount of helpful information given him in regard to cotton culture in the San Joaquin Valley and the Southwest in general, and to H. G. McKeever, of the same office, for invaluable assistance in field observations and the collection of specimens.

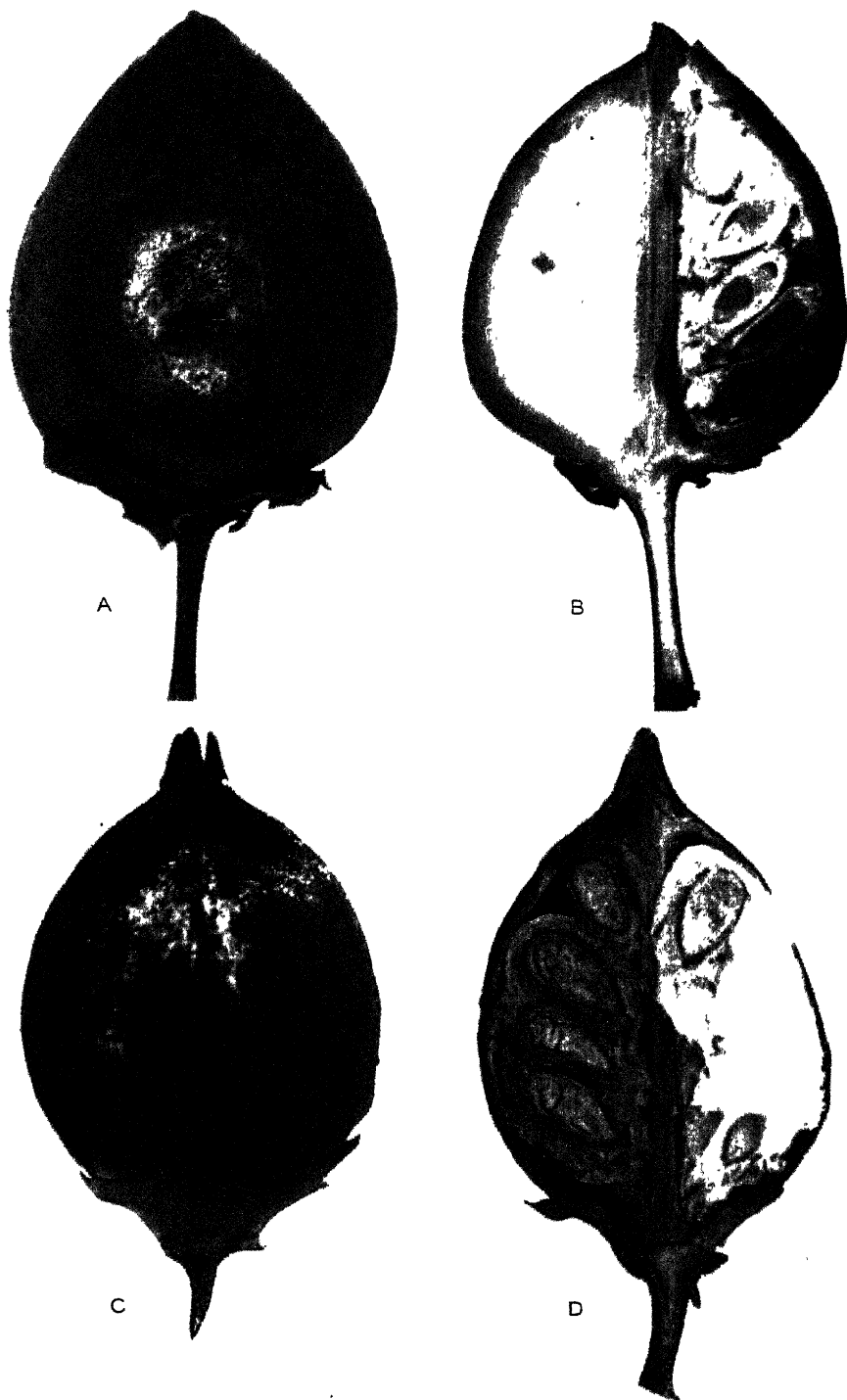
<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 312.

The occurrence of *Aspergillus niger* on cotton bolls was briefly reported by the writer (8) in 1925. Aside from the decaying bolls, the fungus was also frequently isolated from young dying squares, from discolored pedicels, and from lesions on the bracts. It was likewise obtained from stem lesions of young seedlings showing symptoms of sore shin. As far as the observations of the last two seasons (1925 and 1926) are concerned, the *Aspergillus* boll decay was more frequently observed in the Southwest than the *Rhizopus* decay and was most prevalent in southern portions of California, though specimens of it were found also in the San Joaquin Valley, in Mexican plantations adjacent to the Imperial Valley, and in Arizona.

The rot begins as a soft pinkish spot either on the side of the boll or somewhere near its base. The hue and the extent of the pink discoloration vary more or less, but the discoloration is always present and is characteristic of this sort of decay (pl. 1, A). As the lesion increases in size, the color of the older decayed area turns from pink to brown and the original discoloration remains only in the freshly decaying regions—that is, on the border of healthy and diseased tissues. If such a boll is cut open through the affected area the same pink to purplish and red-brown shades of the invaded tissues will be seen (pl. 1, B). The fructification of the fungus begins in relatively early stages of decay, but the spores are white or light gray at first, darken very rapidly, and in a short time give the diseased bolls a “smutty” appearance (pl. 2, B). The fungus is capable of destroying all parts of the boll—the capsule, the lint, and the seeds. Cultures of the organism have been obtained from all such affected tissues. When it is destroyed by the parasite, the entire boll dries up and remains closed (pl. 2, A). Frequently, however, the progress of the decay is checked, either by unfavorable environmental conditions not yet understood, or by the natural development of the boll if it is affected when nearly mature. In such cases the bolls open only partially, the affected portions remaining closed. The black masses of the *Aspergillus* spores may be seen in abundance both on the inside and the outside of the bolls (pl. 2, C and D).

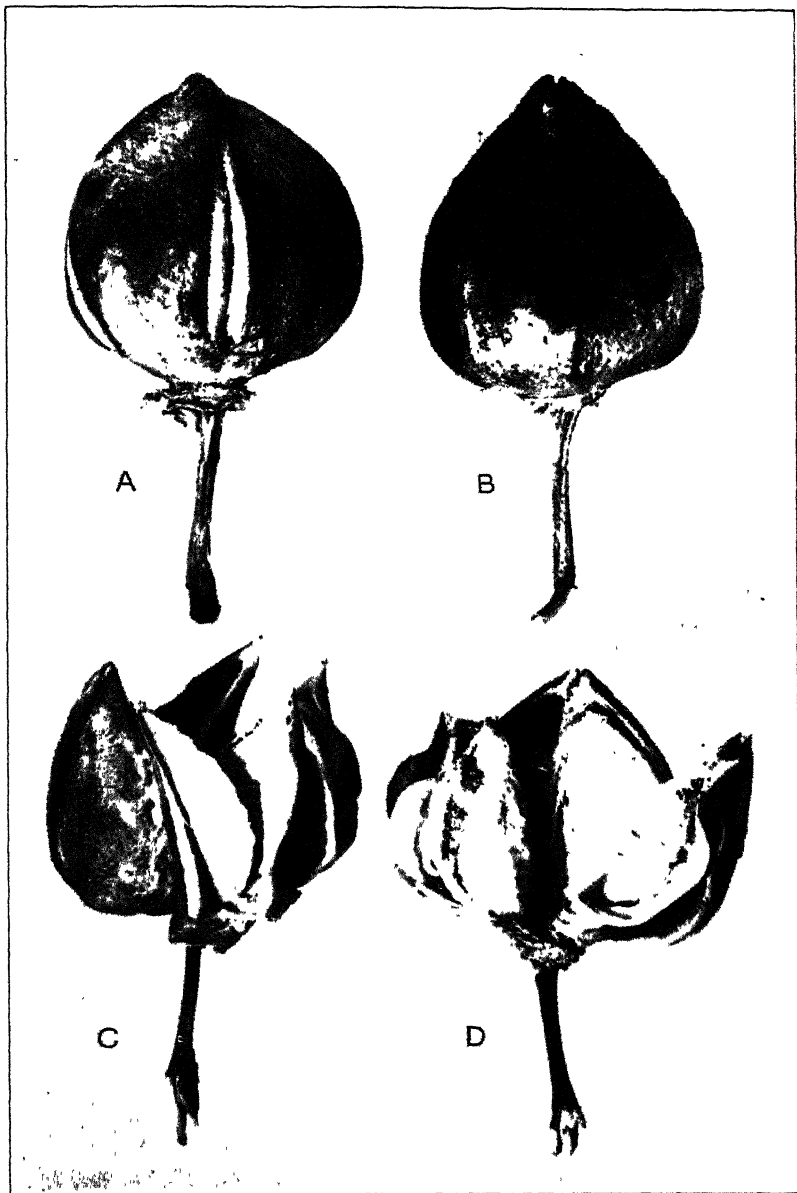
Artificial inoculations with pure cultures of *Aspergillus niger* were made on growing bolls in the field and in the greenhouse, and on picked bolls incubated in moist chambers in the laboratory. The percentage of infection was greatest when picked bolls were used fresh—as soon as they were brought to the laboratory. Bolls which remained in the room for several days before inoculation gave only a small percentage of infection, showing that a certain degree of succulence of the tissues is necessary for the most destructive results. Atmospheric humidity likewise appears to be essential for the best activity of the parasite. This is shown by the fact that the bolls growing in the greenhouse were affected more readily than those growing in the field. The inoculum was introduced partly by applying it to wounds made either with a scalpel or with a needle, and partly by smearing it on the surface of bolls not injured artificially.

With but one exception—namely, in the case of a group of uninjured inoculated bolls placed in a moist chamber (see Table 1)—positive results were obtained only in those cases in which injured bolls were inoculated. One of the bolls placed in the chamber became infected, but proof was lacking that this boll had been brought from the field entirely uninjured. This seems to indicate that the



TWO FORMS OF COTTON-BOLL DECAY





Later stages of cotton-boll decay caused by *Aspergillus niger* Van Tiegh.  
A.—Rapidly decayed and dried-up boll with a very scanty fructification of the fungus.  
B.—Boll destroyed by a slower decay with abundant fructification.  
C. and D.—Partially decayed and partially opened bolls (bracts removed to show larger surface of the bolls).



fungus is mainly if not exclusively a wound parasite. The decay as it naturally occurs in the field often is associated with visible insect wounds, particularly with those made by the bollworm, although in many instances such injuries are not apparent. It is possible, however, that the infection in these cases is associated with some minute punctures caused by smaller insects. In the case of the wound inoculations, the decay did not always appear on the surface at the point of the inoculation, but sometimes developed first within the boll and then broke through at a point more or less removed from the wound. The time necessary for the appearance of the first external signs of the decomposition varied from three to eight days.

A more detailed account of the inoculation tests and their results is given in Table 1.

TABLE 1.—Results of inoculating cotton bolls with *Aspergillus niger*

Group and kind of material used	Time between inoculation and final examination (days)	Inoculated				Controls				Remarks
		Injured		Uninjured		Injured		Uninjured		
		Total number	Number affected	Total number	Number affected	Total number	Number affected	Total number	Number affected	
Group 1: Green picked bolls in moist chambers.	8	11	3	10	0	10	1	10	0	Discoloration characteristic only with a few larger lesions. One boll decayed completely; three bolls decayed mostly in the interior. Most injured inoculations showed decay on the third day, the largest involving three-fourths of the boll and the interior.
	9	14	14	13	0	10	0	-----	-----	
	15	57	55	11	1	12	0	-----	-----	
	Total in group	82	72	34	1	32	1	10	0	87.5 per cent of the injured bolls inoculated became affected.
Group 2: Growing bolls in the greenhouse.	16	4	4	-----	-----	(a)	(a)	(b)	(b)	Typical decay, but not involving more than half the boll; part visible on the third day and the remainder on the eighth day; unaffected portions split and the fungus fruiting appeared on the surface.
	3	4	4	-----	-----	-----	-----	-----	-----	
	Total in group	8	8	-----	-----	-----	-----	-----	-----	100 per cent infection.
	Group 3: Growing bolls in the field.	14	25	19	23	0	25	0	25	0
13		36	32	5	0	17	0	-----	-----	
9		13	12	-----	-----	6	0	-----	-----	
Total in group		74	63	30	0	48	0	25	0	85.1 per cent of infection. Average infection with wound inoculations, 87.2 per cent.
Total in three groups.	164	143	64	1	80	1	35	0		

<sup>a</sup> No injured checks were used.

<sup>b</sup> A number of uninjured bolls growing at the same time remained healthy.

All of the inoculations included in Table 1 were carried on with cultures of *Aspergillus niger* isolated from decaying cotton bolls. In addition, six growing bolls were inoculated with culture No. 1001, previously isolated by the writer (?) from a potato tuber, in order to ascertain the pathogenicity of this culture to cotton and the character of symptoms produced. The inoculations were made with

one of the series included in Group 3. All of the six bolls became infected and the symptoms were identical with those produced by the strains isolated from cotton. The decay developed to the extent of one-half of the boll, the fungus fruiting on the diseased part. The unaffected portions split open in a typical manner.

It has been noted that under natural conditions in the field earlier sets of the bolls show the largest percentage of infection. Inasmuch as the beginning of the decay is often associated with bollworm injuries, the heavier infection in the earlier bolls may readily be explained by the fact that the bollworm, which is subject to many natural enemies, diminishes in numbers as the season progresses.

#### RHIZOPUS NIGRICANS

Unlike *Aspergillus niger*, *Rhizopus nigricans*<sup>4</sup> is a well-known parasite of many cultivated crops (4), though the cotton plant has not been on the list of its hosts until comparatively recently. Its association with the sore-shin lesions in Egypt was suspected by Balls (1, p. 19) to be more than accidental, although in rather extensive inoculation experiments by Briton-Jones (3, p. 6-18) positive results on young seedlings were obtained only with *Rhizoctonia solani*, whereas *Rhizopus nigricans* failed to cause any damage. The same author (2), however, definitely established the fact that the latter fungus causes a decay of bolls in Egypt, gaining entrance through insect wounds. A few years later Kirkpatrick (5) published a detailed account of his studies of this decay, showing the nature of the damage caused and the resulting losses to the growers.

In the United States this disease may be found in the same localities of the Southwest in which the *Aspergillus* rot occurs. However, during the seasons of 1925 and 1926 the former appeared to be less prevalent than the latter, and could be found in greater abundance in the San Joaquin Valley than in sections farther south.

As far as cotton bolls are concerned *Rhizopus nigricans*, like *Aspergillus niger*, appears to be principally, if not exclusively, a wound parasite. In most cases it was found to be associated clearly with the circular holes made by the bollworm. Kirkpatrick (5) from his studies of this disease in Egypt arrived at the definite conclusion that *R. nigricans* is strictly a wound parasite, and he described several insect agencies responsible for bringing about the infection.

The *Rhizopus* decay, as it occurs in the Southwest, is quite distinct through all its stages of development. It lacks entirely the pink discoloration characteristic of the *Aspergillus* decay. The affected portions of the capsule are olive green in color and retain this uniform discoloration until the decayed parts dry up, at which time they become darker. The colored plate accompanying the Briton-Jones paper shows a very dark-brown color of the affected portions of the capsule, which may be observed in this country only at the time when the bolls begin to dry up.

In the earlier stages of the disease the specimens observed by the writer in the United States showed merely a dark-green color of the lesions (pl. 1, C), and little, if any, of the brown or reddish shades. The discoloration of the interior tissues of a freshly decaying boll

<sup>4</sup> L. L. Harter, of the Office of Vegetable and Forage Diseases, Bureau of Plant Industry, examined the writer's culture and confirmed its identification.

is not so uniform and homogeneous and varies from steel gray to slightly purple, with shades of pink and yellow, the latter shades occurring particularly in the seeds (pl. 1, D).

The fruiting stage of the fungus can also be readily distinguished from that of *Aspergillus niger*. The spore masses are not quite so dense as those of the latter organism, and they form a dark-gray or blue-gray rather than a sooty-black powdery film over the boll (compare pl. 1, A, with pl. 1, C, and pl. 2, B). The progress of the decay is somewhat more rapid than that observed in the case of *Aspergillus*, as for example under humid greenhouse conditions.

With respect to other characteristics, the two diseases are very similar. The *Rhizopus* decay may break through at points other than the infection wound and may affect either the entire boll or only a part of it. In the latter case the unaffected part may open up and the fungus fructification be seen on the inside, much in the manner shown for the *Aspergillus* decay. Moreover, it affects not only the capsule but also the immature lint and the seeds, as is clearly evident from the illustrations in Plate 1.

*Rhizopus nigricans* was isolated by the writer also from discolored pedicels, dying small squares, and spots on bracts. It frequently occurs on stem lesions of young cotton seedlings showing symptoms of sore shin, either alone or in association with other fungi. These lesions may be reproduced by means of artificial inoculations with pure cultures of the fungus (9), although not so readily as with *Rhizoctonia solani*.

The results obtained by inoculating cotton bolls with *Rhizopus nigricans* are given in Table 2.

TABLE 2.—Results of inoculating cotton bolls with *Rhizopus nigricans*

Group and kind of material used	Time between inoculation and final examination (days)	Inoculated				Controls				Remarks	
		Injured		Uninjured		Injured		Uninjured			
		Total number	Number affected	Total number	Number affected	Total number	Number affected	Total number	Number affected		
Group 1: Green-picked bolls in moist chambers.	5	12	11	-----	-----	12	0	-----	-----	91.7 per cent infection in Group 1: the outer capsule was destroyed first, then the hard membranes were attacked, but the fiber seemed to remain unaffected.	
Group 2: Growing bolls in the greenhouse.	{	4	3	3	-----	-----	(*)	(*)	(*)	(*)	{ Four inoculated bolls decayed completely, the remainder from one-third to one-half.
		3	3	3	-----	-----	(*)	(*)	(*)	(*)	
		4	6	6	-----	-----	3	0	-----	-----	
Total in group...		12	12	-----	-----	3	0	-----	-----	100 per cent infection in Group 2.	
Group 3: Growing bolls in the field.	{	13	34	18	5	0	17	0	-----	-----	{ Typical <i>Rhizopus</i> decay affecting from a portion to entire boll.
9		12	7	-----	-----	6	0	-----	-----		
Total in group...		46	25	5	0	23	0	-----	-----	54.4 per cent of infection in Group 3.	
Total in three groups.		70	48	5	0	38	0	-----	-----	Average infection with wound inoculations, 68.6 per cent.	

\* No injured checks were used in these cases.

† A number of uninjured bolls growing at the same time remained healthy.

## SUMMARY

Two forms of decay of cotton bolls, frequently referred to as "smut" in their later stages, have been found to occur commonly in southwestern United States.

These diseases are not true smuts and have only a superficial resemblance to the smuts.

One of these forms of decay is caused by *Aspergillus niger* Van Tiegh. and the other by *Rhizopus nigricans* Ehr.

The two diseases may be readily distinguished by the discoloration of the affected tissues as well as by the character of the fruiting stages of the parasites.

Both organisms readily produced rot of artificially wounded and inoculated cotton bolls, but failed to affect uninjured bolls.

The infection in the field apparently depends on injuries caused by various insects, the most noticeable of which are those caused by the bollworm. Control measures, therefore, will have to be directed chiefly against these insect enemies.

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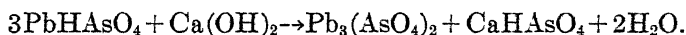
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# A STUDY OF LEAD ARSENATE AND LIME SPRAY MIXTURES<sup>1</sup>

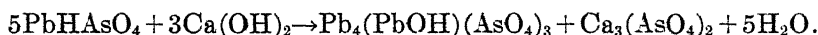
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## INTRODUCTION

For several years the addition of slaked lime to lead arsenate sprays, for the purpose of decreasing or preventing injury to stone fruits, such as peach and plum, has been recommended by the United States Department of Agriculture. The chemical reactions which may take place in such a mixture have received the attention of at least two investigators. Robinson (8)<sup>2</sup> suggests that the reaction which takes place may be represented by the equation:



Campbell (2) on the basis of certain "semiquantitative experiments" proposes that "for the purpose of discussion" the reaction may be represented by the equation:



Experiments carried out in the summer of 1925 indicate that neither of the above reactions represents completely the change which takes place, and it appears desirable, therefore, to give an account of this work.

The first part of the present paper embodies the results obtained in laboratory studies of the chemical reactions which may occur in mixtures of acid lead arsenate and lime. The second part of the paper deals with the effectiveness of the mixture in preventing the arsenical injury which results when lead arsenate is used alone in spraying peach orchards.

## LABORATORY EXPERIMENTS

### EARLIER INVESTIGATIONS

The most comprehensive investigations of the arsenates of lead, carried out in recent years, are those of C. C. McDonnell and C. M. Smith (5) and of McDonnell and Graham (4). Tartar and Robinson (10) and G. E. Smith (9) have also made a study of these compounds.

If we confine our attention to the compounds which can be prepared by precipitation from solution, there are six arsenates of lead to be taken into account. Using the nomenclature of McDonnell and Smith (5), these are: (1) Monolead ortho arsenate,  $\text{PbH}_4(\text{AsO}_4)_2$ ; (2) dilead ortho arsenate,  $\text{PbHAsO}_4$ ; (3) trilead ortho arsenate  $\text{Pb}_3(\text{AsO}_4)_2$ ; (4) 4, 1, 3, 1-lead-hydroxy arsenate (hydroxy mimetite),

<sup>1</sup> Received for publication April 15, 1927; issued October, 1927. Contribution No. 27 from the Japanese Beetle Laboratory, Riverton, N. J.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 321.

$\text{Pb}_4(\text{PbOH})(\text{AsO}_4)_3 \cdot \text{H}_2\text{O}$ ; (5) 5, 2, 4-lead-hydroxy arsenate,  $\text{Pb}_5(\text{PbOH})_2(\text{AsO}_4)_4$ ; (6) octo-lead arsenate,  $8\text{PbO} \cdot \text{As}_2\text{O}_5 \cdot \frac{1}{2}\text{H}_2\text{O}$ .

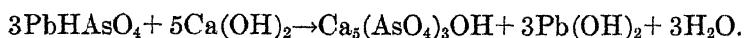
The first of these is obtained only in strongly acid solutions, and is therefore not of importance in the present discussion.

Four arsenates of calcium are described in the literature. These are: (1) Primary calcium arsenate,  $\text{CaH}_4(\text{AsO}_4)_2$ ; (2) secondary calcium arsenate,  $\text{CaHAsO}_4$ ; (3) tertiary (normal) calcium arsenate,  $\text{Ca}_3(\text{AsO}_4)_2$ ; (4) basic calcium arsenate,  $\text{Ca}_5(\text{AsO}_4)_3\text{OH}$ .

The first three of these have been recognized for many years. The existence of the last one seems to have been suspected by several investigators. It has been prepared and studied by Tartar, Wood, and Hiner (11). These investigators have obtained evidence that an arsenate of calcium more basic than  $\text{Ca}_5(\text{AsO}_4)_3\text{OH}$  is not formed by the action of calcium hydroxide solution on this compound at ordinary temperatures.

McDonnell, Smith, and Coad (6) studied the effect of atmospheric carbon dioxide on commercial calcium arsenate during storage. They found that carbonation increased the "water-soluble arsenic oxide," but that when the sample was sufficiently basic, the "soluble arsenic oxide" in the completely carbonated samples exceeded 1 per cent in only one case.

On the basis of the work just discussed, it becomes possible to state what reactions will probably occur in a mixture of acid lead arsenate ( $\text{PbHAsO}_4$ ) and a large excess of calcium hydroxide, suspended in water. There will be formed basic calcium arsenate, as a result of the decomposition of the acid lead arsenate. The lead arsenate will probably go through several stages of basicity, using the term in the sense of ratio ( $\text{PbO}:\text{As}_2\text{O}_5$ ), and may eventually be deprived of all arsenic oxide, leaving one of the hydrates of lead oxide as a final product. Assuming, for the moment, that lead hydroxide ( $\text{Pb}(\text{OH})_2$ ) is the final product we may represent the change which takes place, when the reaction goes to completion, as:



#### EXPERIMENTS IN 1925

##### ACTION OF CALCIUM HYDROXIDE ON ACID LEAD ARSENATE

The lead arsenate used in these experiments was a commercial product which was dried at  $110^\circ \text{C}$ . to constant weight. Its composition was found by analysis to be:

	Found	Calculated for $\text{PbHAsO}_4$
Lead oxide ( $\text{PbO}$ ).....per cent..	64.94	64.30
Arsenic oxide ( $\text{As}_2\text{O}_5$ ).....do....	31.50	33.11
Water ( $\text{H}_2\text{O}$ ) (by difference).....do....	3.56	2.59

The lead arsenate used was therefore slightly more basic and contained somewhat more water than corresponds to the formula  $\text{PbHAsO}_4$ .

A saturated solution of lime water was prepared from c. p. calcium hydroxide, and analyzed, for each series of experiments, by titration with N-100 hydrochloric acid solution, using phenolphthalein as indicator.

The experiment was carried out as follows: A portion of lead arsenate was weighed out accurately and placed in a jar provided with a glass top and rubber seal, to which was added a definite quantity of lime water and freshly distilled water to give the desired dilution. The jar was then sealed and placed in a motor-driven shaking machine. After shaking at room temperature (about 25° C.) for the required time, the contents of the jar were allowed to settle (usually over night) and 10 c. c. of the clear liquid was removed for titration with N-100 hydrochloric acid. The calcium hydroxide remaining in the solution was subtracted from the amount originally introduced. It was assumed that this difference gave the amount of the calcium hydroxide which had reacted with the lead arsenate. In all these experiments the effect of atmospheric carbon dioxide was reduced to a minimum by working rapidly and removing the tops of the reaction vessels just enough to permit the withdrawal of the samples.

In the first series of experiments the reacting substances were brought together and the shaking continued for a sufficiently long time to permit the reaction to go to completion. The results are summarized in Table 1.

TABLE 1.—Action of  $\text{Ca}(\text{OH})_2$  solution on  $\text{PbHAsO}_4$ , after long-continued shaking, at room temperature (25° C.)

Lead arsenate used	$\text{Ca}(\text{OH})_2$ added	$\text{Ca}(\text{OH})_2$ left	$\text{Ca}(\text{OH})_2$ used up	Total volume	Time of shaking	$\text{Ca}(\text{OH})_2$ used up	
						Per gram of lead arsenate	Per gram of $\text{As}_2\text{O}_5$
Grams	Grams	Grams	Grams	C. c.	Hours	Grams	Grams
0.50	0.3180	0.1440	0.1740	200	150	0.3480	1.105
.75	.3290	.0920	.2370	200	150	.3160	1.003
2.00	1.4927	.7945	.6982	1,000	290	.3491	1.108
3.00	1.4927	.5764	.9163	1,000	290	.3054	.969

For the production of  $\text{Ca}_5(\text{AsO}_4)_3\text{OH}$ , 1 gm. of  $\text{As}_2\text{O}_5$  reacts with 1.072 gm. of  $\text{Ca}(\text{OH})_2$ . The other product of the reaction is one of the hydrates of lead oxide.

In two of the jars there was formed a greenish-yellow, flaky substance. This was separated from the white calcium arsenate by stirring, settling, and decantation. The greenish-yellow material, being much heavier, settled quickly. A quantity sufficient for analysis was collected and dried in the air. A determination of lead oxide in the air-dried sample showed it to contain 96.32 per cent  $\text{PbO}$ . This result corresponds closely to the hydrate  $2\text{PbO} \cdot \text{H}_2\text{O}$  (96.12 per cent  $\text{PbO}$ ) which has been described by Luedeking (3). Another hydrate,  $3\text{PbO} \cdot \text{H}_2\text{O}$ , has been described by Pleiszner (7), who obtained it by the action of barium hydroxide on warm solutions of lead salts, or cold solutions of  $\text{PbO}$ . The optical properties of the greenish-yellow crystals obtained in the writers' experiments do not agree well with those ascribed to the hydrate  $2\text{PbO} \cdot \text{H}_2\text{O}$ , and the composition does not agree with the hydrate  $3\text{PbO} \cdot \text{H}_2\text{O}$ .

The results leave no doubt, however, as to the fact that at ordinary temperatures the reaction between lead arsenate and calcium hydroxide, if given sufficient time, results in the complete decomposition of

the lead arsenate, with the formation of basic calcium arsenate and a hydrate of lead oxide.

#### RATE OF REACTION

To ascertain to what extent this reaction proceeds in the ordinary spraying operation a second series of experiments was undertaken, in which weighed quantities of lead arsenate were shaken with known quantities of calcium hydroxide for periods of time which were insufficient for the completion of the reaction. Preliminary experiments having shown that the reaction takes place rather slowly, analysis of the solution was made at the end of 21 and of 37 hours of shaking. The rate of disappearance of calcium hydroxide from the solution may be taken as a measure of the rate of the reaction. The results are given in Table 2.

TABLE 2.—*Effect of shaking 1 gram of lead arsenate with 200 c. c. of a solution of calcium hydroxide (temperature about 25° C.)*

Quantities of Ca(OH) <sub>2</sub> (in milligrams)—				
Originally present	Used up in 0 to 21 hour period	Used up per hour in 0 to 21 hour period	Used up in 21 to 37 hour period	Used up per hour in 21 to 37 hour period
110.9	29.4	1.40	32.5	2.03
126.7	30.2	1.44	28.5	1.78
129.8	31.0	1.47	24.5	1.53
133.0	30.2	1.44	24.1	1.50
136.0	28.8	1.37	25.1	1.57
142.3	27.8	1.32	23.0	1.44

It is evident that the rate of disappearance of calcium hydroxide does not vary greatly with time, and that it is exceedingly slow. The complete decomposition of 1 gm. of the lead arsenate used in these experiments requires about 338 mgm. of calcium hydroxide. The average hourly rate of 1.52 mgm. corresponds to the complete decomposition of less than one-half of 1 per cent of the lead arsenate.

On the basis of these results it seems reasonable to conclude that the spray which reaches the foliage consists essentially of unchanged lead arsenate particles, possibly altered superficially to basic calcium arsenate and either lead arsenate or lead hydroxide, and this is mixed with a considerable amount of slaked lime. After such a mixture is exposed to the air, carbonation takes place, so that a deposit of the composition mentioned will soon be altered completely to a mixture of calcium carbonate, acid lead arsenate, and a small amount of basic lead arsenate, or calcium arsenate.

#### RATE OF LIBERATION OF "SOLUBLE ARSENIC"

In a final series of experiments the question of whether the action of atmospheric carbon dioxide results in any large increase in "soluble arsenic" was taken up. The lead arsenate used in these experiments was somewhat basic to begin with, and had a low content of "soluble arsenic." If the chemical action of the lime, followed by atmospheric action, tended to increase the content of "soluble arsenic" appreciably, this should be readily apparent.

In these experiments 2 gm. of lead arsenate were accurately weighed, mixed into a paste with water, and then the cooled milk of lime resulting from the action of about 40 c. c. of water on 6 gm. of quicklime was added. The whole was intimately mixed on a watch glass and allowed to evaporate to dryness in the air. As soon as the material was dry it was powdered in a mortar and then returned to the watch glass, where it was exposed to the air of the laboratory. A number of such samples were prepared and a determination of "soluble arsenic" by the usual method (1, p. 50) was made at intervals of several days. The results are as follows:

Sample No.	Time of exposure (days)	Per cent of soluble arsenic, expressed as $As_2O_5$
1.....	1	0.27
2.....	3	.44
3.....	5	.60
4.....	8	.60
5.....	9	.68
6.....	16	.70
7.....	23	.64
8.....	154	.65
Lead arsenate without lime.....		.44

The solutions obtained by treating the above samples with water were tested with phenolphthalein to determine whether the lime had been completely carbonated. All samples gave an alkaline reaction, even the one which had been exposed to the air for 154 days.

In order to determine what the result would have been, had complete carbonation taken place, two additional experiments were carried out. In one of these the water suspension obtained from sample No. 8 was rendered just acid to phenolphthalein by bubbling carbon dioxide through it. The "soluble arsenic" was then determined in the usual manner. Since it was very difficult to avoid a slight excess of the gas, the second experiment consisted in adding 11 gms. of c. p. calcium carbonate powder to 2 gms. of the lead arsenate and 1 liter of carbon-dioxide-free water. The "soluble arsenic" was then determined as before, the results being as follows:

	Per cent soluble arsenic expressed as $As_2O_5$
Sample 8, acidified with $CO_2$ gas.....	4.15
Lead arsenate treated with c. p. $CaCO_3$ .....	3.15

The results show that, under the conditions of the experiment, carbonation takes place very slowly, and that as long as there is any unchanged calcium hydroxide the amount of "soluble arsenic" is very low. As soon as all the lime has been converted to carbonate, however, there is a large increase in the "soluble arsenic."

#### ORCHARD EXPERIMENTS

The results of the laboratory work just described would seem to indicate that the addition of lime to lead arsenate should be effective in preventing injury to peach foliage for a time at least. During the period from the time the spray application is made until the calcium

oxide, not combined as arsenate, is carbonated, the proportion of the "soluble arsenic" is low, and a corresponding freedom from injury may be expected. To determine this point, and also to obtain data on the efficiency of various spray combinations of acid lead arsenate and slaked lime against the Japanese beetle, a number of experiments were conducted in peach orchards in the vicinity of Riverton, N. J., in 1923, 1924, 1925, and 1926.

All of the orchards used for the experimental work consisted of bearing trees in a healthy condition, with a very good growth of foliage. The sprays were delivered by a power sprayer at a pressure of 200 pounds per square inch. Disc nozzles were used with rods. A thorough application was made. In the experiments reported for 1923, 1924, and 1926 the lead arsenate and lime were used in connection with orchard control measures against the Japanese beetle. The lead arsenate and lime were commercial products and no chemical analyses were made of their composition. In 1925, however, a special study was made of the effect of lime and lead arsenate mixtures on peach trees, and chemical analyses were made of the lime and lead arsenate used during that year.

Observations were made at intervals throughout the summer for injury to the fruit, foliage, and wood. The injury, expressed in percentage, is an estimate of the area injured as compared with the rest of the tree. The characteristics of arsenic injury were definite in every instance in which record was made as to the extent of injury.

The experiments made and the results obtained each year are given below.

#### EXPERIMENTS PERFORMED IN 1923

The orchard selected for these experiments consisted of the Rochester variety of peaches. Twenty-five trees were selected for each plot and these plots were sprayed June 29. The treatment of the plots was as follows:

- Plot 1. Lead arsenate, 3 pounds; water, 50 gallons.
- Plot 2. Lead arsenate, 2 pounds; water, 50 gallons.
- Plot 3. Lead arsenate, 1 pound; water, 50 gallons.
- Plot 4. Lead arsenate, 3 pounds; lime, 3 pounds; water, 50 gallons.
- Plot 5. Lead arsenate, 2 pounds; lime, 3 pounds; water, 50 gallons.
- Plot 6. Lead arsenate, 1 pound; lime, 3 pounds; water, 50 gallons.
- Plot 7. Control, lime, 3 pounds; water, 50 gallons.
- Plot 8. Control unsprayed.

#### RESULTS

Observations were made throughout the summer for fruit, wood, and foliage injury, the final results being recorded September 26. At this time heavy deposits of spray residue remained on the fruit and with the exception of plot 1 no foliage, fruit, or wood injury was evident. Plot 1, which had been treated with 3 pounds of lead arsenate to 50 gallons of water, showed foliage injury to the extent of approximately 10 per cent. Aside from this, the trees in all of the plots were vigorous and of good color.

## EXPERIMENTS PERFORMED IN 1924

The orchard selected consisted of the following varieties: Hiley Belle, Carman, Old Nixon, and Elberta. The plots consisted of trees of each variety. The treatments were applied June 12, as follows:

- Plot 1. Lead arsenate,  $1\frac{1}{2}$  pounds; lime, 3 pounds; water, 50 gallons.
- Plot 2. Lead arsenate,  $1\frac{1}{2}$  pounds; water, 50 gallons.
- Plot 3. Control, unsprayed.
- Plot 4. Lead arsenate, 2 pounds; lime, 3 pounds; water, 50 gallons.
- Plot 5. Lead arsenate, 2 pounds; water, 50 gallons.
- Plot 6. Control, unsprayed.
- Plot 7. Lead arsenate, 1 pound; lime, 3 pounds; water, 50 gallons.
- Plot 8. Lead arsenate, 1 pound; water, 50 gallons.
- Plot 9. Control, unsprayed.
- Plot 10. Lead arsenate,  $\frac{1}{2}$  pound; lime, 3 pounds; water, 50 gallons.
- Plot 11. Lead arsenate,  $\frac{1}{2}$  pound; water, 50 gallons.

## RESULTS

Observations were made at various periods during the season, final results being recorded August 31. Plot 2, treated with lead arsenate  $1\frac{1}{2}$  pounds, water 50 gallons, and plot 5 treated with lead arsenate 2 pounds, water 50 gallons, showed foliage injury to the extent of 10 per cent, and slight wood and fruit injury. All other plots were free from arsenical injury. Thus it is shown that the lime added to lead arsenate prevented injury to the foliage in plots 1 and 4.

## EXPERIMENTS PERFORMED IN 1925

A 4-year-old peach orchard, planted especially for experimental work by the Japanese Beetle Laboratory, in 1921, consisting of Carman, Hiley Belle, Georgia Bell, and Elberta, was selected. Stone lime<sup>4</sup> from three different manufacturers was used. Three applications of lead arsenate and lime were made May 12, June 12, and July 13, respectively, as follows:

- Plot 1. Lead arsenate,  $1\frac{1}{2}$  pounds; lime from Bellefonte, Pa., 3 pounds; water, 50 gallons.
- Plot 2. Lead arsenate  $1\frac{1}{2}$  pounds; lime from Norristown, Pa., 3 pounds; water, 50 gallons.
- Plot 3. Lead arsenate,  $1\frac{1}{2}$  pounds; lime from Sherwood, Tenn., 3 pounds; water, 50 gallons.
- Plot 4. Lead arsenate,  $1\frac{1}{2}$  pounds; water, 50 gallons.
- Plot 5. Control, unsprayed.

The composition of the lime used in these experiments is as follows:<sup>5</sup>

	Lime from Bellefonte	Lime from Norristown	Lime from Sherwood
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
SiO <sub>2</sub> .....	0.72	0.29	0.43
Al <sub>2</sub> O <sub>3</sub> , Fe <sub>2</sub> O <sub>3</sub> .....	.61	.59	2.50
CaO.....	95.37	97.07	55.46
MgO.....	.24	.26	35.18
Loss on ignition.....	2.48	1.13	6.49

<sup>4</sup> The authors are indebted to G. J. Fink, chemical director of the National Lime Association, Washington, D. C., through whose courtesy the lime was furnished.

<sup>5</sup> The composition of the lead arsenate used is given on page 2.

## RESULTS

The results from the applications of sprays were very definite. Although a treatment consisting of three applications of lead arsenate at the rate of  $1\frac{1}{2}$  pounds to 50 gallons of water is somewhat in excess of the usual recommendations, the writers used this in order to be sure that the effect upon the trees of adding the lime to the lead arsenate would be pronounced. The trees in plot 4, which had been treated with  $1\frac{1}{2}$  pounds of lead arsenate to 50 gallons of water, were 90 per cent defoliated August 31, when the final results were recorded. The wood on these trees was so severely injured that only 2 trees of the 24 in the plot were living in 1926. The foliage in plot 3 was injured 5 per cent. All other plots were free from arsenical injury. Apparently a lime high in magnesia is as satisfactory for this purpose as one low in magnesia.

## EXPERIMENTS PERFORMED IN 1926

The same orchard that was used in 1924 was again used in 1926 for conducting these studies. Four plots were used to determine the effect on the foliage of adding lime to lead arsenate, and the treatment was as follows:

- Plot 1. Lead arsenate,  $1\frac{1}{2}$  pounds; lime, 3 pounds; water, 50 gallons.
- Plot 2. Lead arsenate,  $1\frac{1}{2}$  pounds; lime, 6 pounds; water, 50 gallons.
- Plot 3. Lead arsenate,  $1\frac{1}{2}$  pounds; water, 50 gallons.
- Plot 4. Control, unsprayed.

## RESULTS

In plot 3, where lead arsenate had been applied without the lime, 10 per cent injury resulted to the foliage, whereas no foliage injury resulted in the plots treated with the lime and the lead arsenate. The results are similar to the results from experiments conducted during previous years, as described above.

The influence of the weather conditions on the physical and chemical action of the lime and lead arsenate mixture is not considered here, but will be discussed in another paper.

## SUMMARY

The action of a solution of calcium hydroxide on acid lead arsenate has been studied in the laboratory.

Under the conditions of the experiments, long-continued action of an excess of calcium hydroxide on acid lead arsenate resulted in decomposition of the lead arsenate, with the formation of basic calcium arsenate and a hydrate of lead oxide.

The reaction takes place slowly, and, under the conditions usually met with in spraying operations, probably less than 1 per cent of the acid lead arsenate is decomposed.

The action of carbon dioxide of the air on the mixture of calcium hydroxide and lead arsenate does not produce an appreciable increase in "soluble arsenic" until after all the lime has been carbonated. Thereafter the amount of "soluble arsenic" increases very materially. In the particular case studied, the increase was from 0.65 per cent to 3.15 per cent of  $\text{As}_2\text{O}_5$ .

Orchard experiments with lead arsenate, with and without the addition of slaked lime, have been conducted during the seasons of 1923 to 1926, inclusive, at Riverton, N. J.

It is definitely shown that slaked lime added to lead arsenate prevents peach foliage injury by "soluble arsenic." In one series of experiments, in which a lime high in magnesia was used, no injury resulted.

There is a practical advantage in combining 3 pounds of slaked lime with  $1\frac{1}{2}$  pounds of acid lead arsenate in 50 gallons of water.

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# THE PINE TIP MOTH IN THE NEBRASKA NATIONAL FOREST<sup>1</sup>

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## INTRODUCTION

The old adage to the effect that hindsight is clearer than foresight applies in forestry as in every other line. If this were not true, the pine tip moth would probably not now be engaging our attention as a pest in the Nebraska National Forest. If the pioneer workers had seen as clearly into the future as we see into the past, forest-pulled planting stock would never have been collected in distant localities for planting in Nebraska. All the trees used in establishing forest growth in the treeless sand-hill region would have been grown from seed, the introduction of the pine tip moth, *Rhyacionia (Evetria) frustrana* (Comstock), var. *bushnelli* Busck, into the Bessey plantations would probably have been avoided, and one of the most serious problems in connection with the growing of trees in the Nebraska National Forest might never have arisen.

Perhaps this is too strong a statement in view of the fact that there is no definite record of exactly when and in what manner the tip moth reached the Nebraska plantations, but the weight of evidence points strongly toward the probability of its introduction with planting stock shipped in from other localities. Several facts support this assumption. It is known that this insect never voluntarily takes wing even during moderately strong winds and that when it does fly it stays close to the ground, where it can quickly reach shelter. Inasmuch as this habit reduces to a minimum the chance that the moth might have been blown into the plantations by high winds, it is unlikely that the moth could have traveled successfully the distance of 50 or 60 miles from the native pine. On the other hand, it is known that forest-pulled jack pine from Minnesota and possibly from other Lake States, and western yellow pine from the Black Hills, where the tip moth is a common but not a serious pest, were used in some of the earlier plantings in Nebraska. The assumption that the insect was introduced with some of these trees, either as eggs, larvae, or pupae, therefore appears to be justified.

The tip moth appeared in the plantations at Halsey, Nebr., in sufficient numbers to attract attention in the year 1909. Since that time it has found its way into practically all the privately owned plantations in the Kinkaid district. Presumably the insect has been distributed to these plantations in nursery stock from the Bessey nursery, at Halsey.

Each year since 1909 the moth has increased in importance in the plantations. It is responsible for killing some trees by repeated injury year after year, and has retarded growth in the other trees.

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<sup>1</sup> Received for publication May 31, 1927; issued October, 1927.

Some trees are barely holding their own against the attack of the pest, while others have been able to make some growth in spite of it.

This is quite a different situation from that existing in most other localities where the tip moth is found. Usually, except in the South where several generations probably occur, the insect is of little economic consequence. Occasionally, however, it becomes epidemic, as in the instance on Nantucket Island reported by Scudder in 1876.<sup>2</sup> From his description it may be concluded that the pest was exceedingly abundant at that time. He states that nearly every new shoot on the infested trees examined had been mined and killed. Since 1876 local outbreaks of a sporadic nature have occurred from time to time in the New England and Atlantic States, but none have been so extensive or serious as the one first described. In fact, aside from this one instance, the pest has attracted little attention. During 1925, however, the moth became abundant on Cape Cod, and another outbreak comparable in proportion and severity to the Nantucket epidemic is now under way.

The tip moth is present generally throughout the Lake States and the Black Hills, but it has never become sufficiently numerous to attract much attention. Its favorite host in the Lake States is jack pine, *Pinus banksiana* (*divaricata*), and in the Black Hills western yellow pine, *Pinus ponderosa*. Throughout the South and West the pest is also widely distributed and is injurious to young hard pines in the large seedling and small sapling stages, but seldom seriously injures trees beyond the sapling stage.

Throughout its range the pine tip moth is characterized by its sporadic outbreaks. One year it will be abundant in certain groves, and perhaps the next year these groves will be almost free from attack, while others in the same general locality will be infested. Even the historic Nantucket outbreak was more or less sporadic in character, inasmuch as the insect was unusually abundant for only a few years and then returned to normal numbers. The factors involved in bringing about these fluctuations are not known. Perhaps the fluctuations are due to variations in the weather, or to fluctuations in the number of the natural enemies of the tip moth, or more probably to a combination of these and other factors.

Why this insect, which normally occurs in such small numbers as to be comparatively unimportant, should be injurious year after year in Nebraska is not clear. Presumably it is due to the fact that some of the environmental factors that hold the pest in check in the native pine forests are lacking in the Nebraska environment. But what these factors are and how they affect the insect are not fully known. It is hoped that further investigations may throw more light on these phases of the problem and that these investigations may lead to the development of effective and economical control.

#### PREVIOUS INVESTIGATIONS

The tip moth has been under intermittent investigation in Nebraska since 1909, when the forest supervisor of the Nebraska National Forest reported its presence to the State entomologist and to the Bureau of Entomology of the United States Department of Agri-

<sup>2</sup>SCUDDER, S. H. THE PINE MOTH OF NANTUCKET—*RETINIA FRUSTRANA*. 20 p., illus. Boston, 1883. (Mass. Soc. Prom. Agr. Pub.)

culture. In the succeeding years up to 1912 observations on the insect were made by representatives of the State entomologist of Nebraska. Much progress was accomplished in studying the biology of the pest. At the end of the season of 1912 the State discontinued the active prosecution of the project. Unfortunately, the results of this work have never been published.<sup>3</sup> No further work was done on this problem until in 1923 the pest was again called to the attention of entomologists by the United States Forest Service. This time representatives of the Bureau of Entomology, United States Department of Agriculture, undertook the investigation in cooperation with the Forest Service. A preliminary survey of the problem was made in 1924. In April, 1925, intensive work on the problem was instituted. The work of the past two seasons has given a fairly complete knowledge of the life history of this pest, and it is the purpose of this paper to present the life history of the tip moth and the present status of the problem. This paper is the product of the above-mentioned cooperative arrangement with the Forest Service. Further investigations concerning the ecology and control of the tip moth are now under way.

#### CHARACTER OF INJURY AND SUSCEPTIBILITY OF SPECIES

The pine tip moth mines and kills the expanding buds and the new growth of almost all of the two or three needled pines. It is obvious that when this type of injury is severe the growth of the trees will be much checked. If heavy injury of this sort occurs year after year on the same trees, the result will be a stooling of the tips, which will produce a dwarfed and deformed tree. This causes, during the large seedling and small sapling period, a very material reduction in the rate of height growth of the forest. Injury of this kind seldom results in the death of a tree, although in some cases, when practically all of the tips have been destroyed year after year, trees occasionally have been killed.

All species of pines in the plantations do not appear to be equally susceptible to this pest. Western yellow pine, *Pinus ponderosa*, is the most severely injured. Norway pine, *Pinus resinosa*, jack pine, *Pinus banksiana (divaricata)*, Scotch pine, *Pinus sylvestris*, and Austrian pine, *Pinus nigra (austriaca)*, follow in order of susceptibility, judging from counts made in 1925 and 1926, which are shown in Table 1 and represented graphically in Figure 1.

TABLE 1.—Damage done by the pine tip moth to different species of pine

Species of pine	Number of trees	Percentage of terminal tips injured		Percentage of lateral tips injured	
		1925	1926	1925	1926
Western yellow.....	200	65.5	97	45.5	71.0
Norway.....	200	.....	96	.....	64.4
Jack.....	200	54.5	80	8.0	18.2
Scotch.....	100	38.0	62	9.4	15.3
Austrian.....	100	.....	3	.....	3.8

<sup>3</sup> The authors are indebted to Myron H. Swenk, State entomologist of Nebraska, for his courtesy in permitting the examination of the notes in his files concerning this insect. At his request, none of the data contained therein have been used in this paper, but a knowledge of the work that had already been done was of considerable assistance in the early stages of the present investigation.

The data in Table 1 are comparable, inasmuch as the trees are all of a highly susceptible size and age. Each year's counts were made on the same plots. The injury to the tips varied from a hollowing of the buds to a mining of the entire new growth.

If it were not for the fact that from the silvicultural point of view the western yellow pine is the tree best suited for sand-hill planting, a substitution of the apparently more resistant Scotch and Austrian pines might well form the basis for tip-moth control; but because of the desirable characteristics of the yellow pine for this sand-hill region, the preservation of that species is to be desired.

Not only does the amount of tip-moth injury vary with the species of pine, but it also varies with the size of the tree. During the first

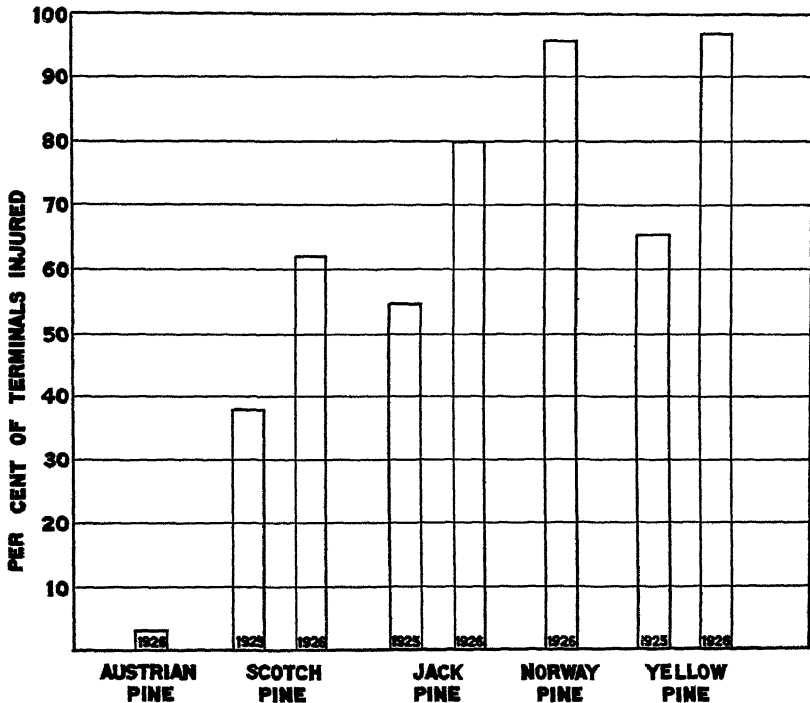


FIG. 1.—Comparison of percentages of terminal tips injured in different species of pine in 1925 and 1926 by *Rhyacionia frustrana bushnellii*

few years after the trees are set out in the plantations very few of them are attacked. This may be because the moths spread slowly from the old plantations into the new ones, or, what seems more probable, because small trees during the first few years are protected more or less by the growth of grass surrounding them. After the trees get above the grass, they become subject to attack and remain highly susceptible until they reach a height of 8 to 9 feet, after which they become less and less susceptible as they increase in height. The taller the trees become, the less likely is the terminal bud of the leading shoot to be destroyed. On the tallest trees in the plantation the greatest injury is done to the lower parts of the trees, whereas the smaller trees are just as likely to have the leading shoot destroyed as to have the laterals attacked.

## SEASONAL HISTORY AND HABITS

Taxonomically the tip moth found in Nebraska is considered to be a geographic variety of the Nantucket pine tip moth, since it does not diverge sufficiently from the eastern form to justify its separation as a distinct species. There are, however, biologic differences, one of which is very striking. The outstanding difference is in the winter habits of the two forms. The eastern variety passes the winter in the pupal stage within the injured pine tips. The Nebraska form, on the other hand, winters as a pupa in the soil beneath the trees. This habit is also characteristic of the tip moth in Minnesota and in the Black Hills.

The moths emerge in early spring, the date of emergence varying from year to year, depending upon the weather. Emergence usually begins early in April and lasts for several weeks. The moths remain active in the plantations until the middle of May or the first of June. They are not rapid fliers and have never been observed flying high above the ground. They spend a large part of their time hidden among the needles of the pine trees, and although they may be very abundant it is unusual to observe many in flight at any one time during the day. About dusk, however, on still, warm evenings a general flight may occur. The moths are seldom observed in voluntary flight in cool or windy weather. So far as known they do not fly at night, or if they do fly at that time, they do not appear to be attracted to light. During the flight period the moths can be shaken from almost any tree; as many as a dozen or more moths can sometimes be shaken from a single small tree. When they are forced to fly under conditions in which they would not voluntarily take wing, they seek the nearest shelter either by flying into a near-by tree or by dropping to the ground. On the ground they often feign death. When at rest on the tree they are very inconspicuous, because their color and shape blend almost perfectly with their surroundings. One of these moths when at rest may easily be mistaken for a bit of dead needle or brown bark. Because of these characteristics they are difficult to observe.

Shortly after the moths emerge oviposition begins. The eggs are about one-half millimeter in diameter, circular in shape, flattened, and light yellow in color. They are usually laid singly, but occasionally in groups of two or three. They are deposited either on the needles, the leaf sheaths, the buds, the bud scales, or the tips; the usual place, however, is on the inner side of a needle. Their size, form, and color would tend to make them inconspicuous, and when it is considered that they are deposited singly it is easy to understand why it is difficult to find them in the field. Oviposition takes place either during the night or early evening. The number of eggs which a moth is capable of laying was not determined in the field. In the laboratory, however, as many as 65 eggs were deposited by a single female. In nature this number might be increased materially. In the laboratory a single moth continued to oviposit throughout a period of one week. The period during which eggs may be found in the field extends from early April into June.

The period of incubation undoubtedly varies considerably, depending upon the weather. During the spring of 1925 the moths had been active and ovipositing for 40 days before the first larva was found in

the field. This would indicate a rather long period of incubation during the cool weather that is characteristic of the spring months. In the laboratory, where the temperature varied from 60° F. at night to slightly above 70° F. during the day, the period of incubation was from 10 days to 2 weeks. The summer brood had a very much shorter period of incubation. At that time the temperature in the laboratory ran between 80° and 90° F. in the daytime and around 70° at night, and the period of incubation varied between 6 and 10 days.

As incubation proceeds the appearance of the eggs changes somewhat. Toward the end of the period the developing embryo may be clearly distinguished through the transparent shell. The eggs are attacked by at least one parasite. The parasitized eggs appear black and opaque instead of yellow. In hatching, the larva breaks through the shell and leaves a rough, irregular opening. In contrast to this an eggshell from which a parasite has emerged can be easily distinguished by the regularly shaped, circular opening through the shell that the adult parasite cuts when it emerges.

When the larvae hatch they are very tiny, about a millimeter and a half in length, with a yellowish-colored body and a black head broader than the body. Their color changes very little throughout the larval period. In the laboratory these young larvae may survive for 24 hours or more without food. Thus, after hatching, a larva has ample time to find a suitable location for its feeding activities. The young larva soon after hatching spins over the spot where it is feeding a thin protective web, which it coats with resin. This web is always located on a new tip, usually at the base of a needle or the base of a bud. Sometimes the larva works on the surface of the stem beneath its web, whereas in other instances the newly hatched larva may tunnel into the base of a needle fascicle and there feed for a short period. Later it burrows either into a bud or into the succulent growth of a new tip. Here it continues to mine until it has completed its development. The web mentioned above may be extended around the base of the bud or along the base of several needle clusters as the larva increases in size. Much of the frass produced during the developmental stage is cast out of the burrow. Thus the opening is kept clear so that the larva can pass in and out freely.

The larvae, so far as observed, always remain either in their tunnels or beneath the protecting web at the opening of the tunnel. When driven from their burrows, however, they may drop by a silken thread. This is particularly true of young larvae. When placed on a new tip or when the web covering is destroyed, the larva very soon spins a new web above itself.

Because of the mechanical difficulties involved in observing individual larvae throughout their developmental period, it was found impossible to observe directly the number of instars through which this insect passes. By measurement of the head capsule of larvae of all sizes collected in the field, however, it was found that they fall into six groups, each of which probably represents an instar. These data, however, were not as conclusive as might be desired because of the intergrading of the groups. There is considerable variation in the size of the head capsule even in the same instar. This is probably due to variations in the abundance or kind of food.

Under the most favorable conditions the length of the larval stage appears to be not less than three weeks. This period may be length-

ened under more adverse conditions. The larvae of the first generation may be found in the field from the middle of May to the first part of July. When fully developed, each larva of the spring generation spins a light silken cocoon inside its burrow and there passes through the pupal stage. On the basis of both laboratory and field observations, the pupal period required two weeks during the season 1925. In 1926, however, in the laboratory 14 to 19 days were required for the pupal period. Pupae of the first generation may be found in the field during the latter two-thirds of June and into July.

Adults of the summer generation begin to emerge during the latter part of June. The period of flight of this generation is comparatively short and lasts less than a month. These moths apparently lay eggs very soon after emergence.

Owing to greater abundance, the summer generation causes much greater injury to the trees than the spring generation. The habits of the larvae, however, are practically the same. They may be observed in the field throughout the greater part of July and August.

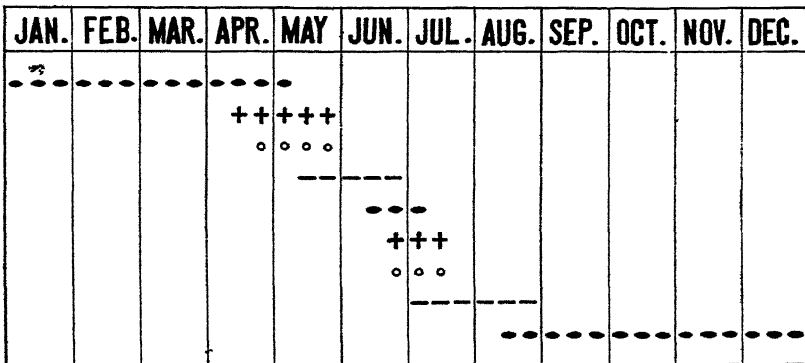


FIG. 2.—Seasonal history of *Rhyacionia frustrana bushnelli* in Nebraska: Solid ovals represent pupal stage; plus signs, adult stage; small circles, egg stage; broken lines, larval stage

Cocooning commences with the oldest larvae about August 10 to 15, and by the end of August all of the larvae have spun cocoons. As has been pointed out before, the cocoons of the second generation are not spun in the tips, as is the case with the spring generation, but the larvae, when full grown, drop to the ground, and in the litter under the trees or just beneath the surface of the sand spin their thin cocoons and transform to pupae. The cocoons spun by the summer generation in the sand seem somewhat heavier than those spun in the tips by the first-brood larvae. This difference, however, may be more apparent than real, because the larvae in spinning the cocoons work in with the threads of silk grains of fine sand. This tends to weight the cocoons and makes them appear heavier than they really are. This mixing of sand with the cocoon makes them much less conspicuous than they would otherwise be, and as a result the cocoons, although abundant, are difficult to find.

Figure 2 illustrates graphically the seasonal history of the tip moth in Nebraska. Figure 3 gives a general idea of the appearance of various stages of the pine tip moth.

In the eastern part of the United States the tip moth also has two generations, but in the Black Hills and in Minnesota only one generation occurs. In both of the latter localities the larvae, after completing their growth, drop to the ground and there spin their cocoons. In the South it is quite possible that more than two generations may occur.

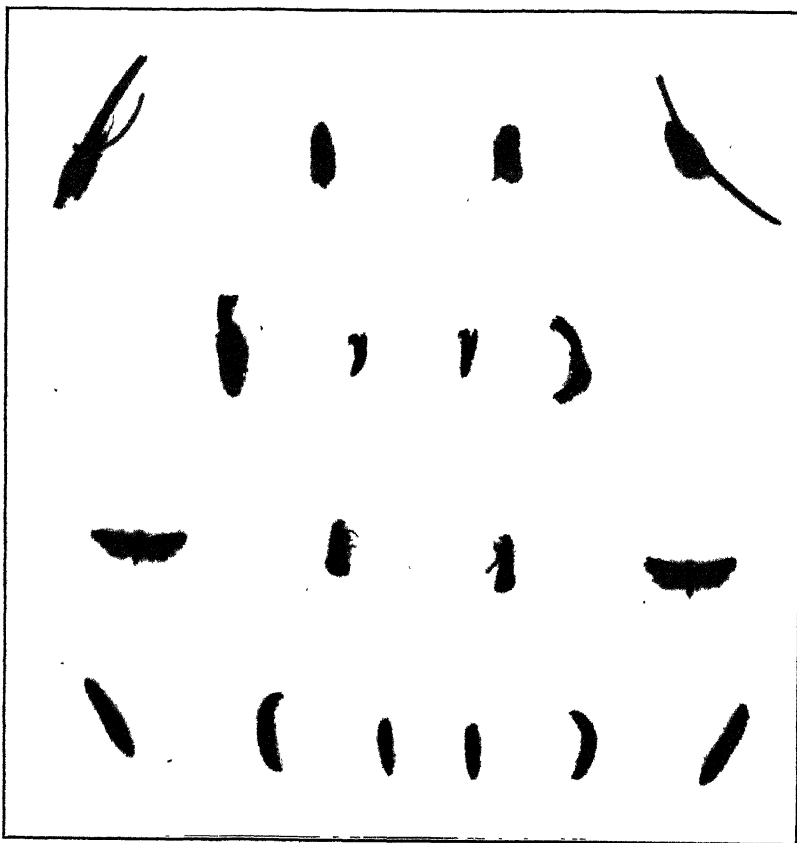


FIG. 3.—*Rhyacionia frustrana bushnelli*: First row (at top), cocoons spun loose in the sand and attached to litter; second row, empty pupal skins, two protruding from cocoons; third row, moths with wings spread and with wings folded; fourth row, four full-grown larvae, and two pupae (in center)

#### PARASITES OF THE PINE TIP MOTH NOW PRESENT IN THE PLANTATIONS

When this investigation of the tip moth in the Bessey plantations was first undertaken it was suspected that one of the reasons for the unusual destructiveness of this pest in Nebraska was the absence of its natural enemies in that region. It is possible that this is true and that the most effective enemies are absent, but observations have shown that at least some parasites are working upon this pest in the plantations.

At the time of the introduction of the tip moth a few of its parasites may have been brought in with it, some may have been acci-

dentally blown into the area, and some of the general feeders, native of the region, may have adopted the tip moth as a host. At least 16 species of parasites have been reared from the tip-moth larvae in the Bessey plantations. Of these, 6 are Ichneumonidae, 3 Braconidae, 5 Chalcidoidea, 1 Bethylidae, and 1 Tachinidae. Two chalcidoid species are the most numerous local parasites. One of these is a member of the genus *Eurytoma*; the other belongs to the genus *Haltichella*. Next in abundance are two species of the genus *Microbracon*. In addition to these parasites of the larva, there is at least one undetermined species of egg parasite, a member of the Chalcidoidea. A list of these parasites that have been determined is as follows:

## Bethylidae:

*Goniozus longiceps* Kief.

## Ichneumonidae:

*Pristomerus*, n. sp.

*Cremastus epagoges* Cush.

*Cremastus* sp.

*Atrometus* sp.

*Epiurus aplopappi* Ashm.

*Itoplectis conquisitor* Say.

## Braconidae:

*Microbracon gelechia* Ashm.

*Microbracon mellitor* Say.

*Microbracon variabilis* Prov.

## Chalcidoidea:

*Eurytoma tylodermatis* Ashm.

*Elasmus setoscutellatus* Cwfd.

*Perilampus chrysopae* Cwfd.

*Habrocytus* sp.

*Haltichella*, n. sp.

## Tachinidae:

At least one unidentified species.

In 1925 infested tips of jack pine and western yellow pine were collected and the moths and parasites were allowed to emerge in the laboratory. In 1926 rearings were made from Norway pine and Scotch pine also. Tables 2 and 3 summarize the results of these rearing experiments.

TABLE 2.—Rearings of tip-moth parasites from local pine tips (spring generation, 1925)

Tree species	Number of tips	Number of moths	Number of parasites	Approximate percentage of parasitism
Western yellow pine.....	600	542	68	11
Jack pine.....	200	110	66	38

From Table 2 it will be seen that the average percentage of parasitism based on the total rearings from yellow pine in 1925 was 11 per cent, while in jack pine it amounted to 38 per cent.

TABLE 3.—Rearings of tip-moth parasites from local pine tips (spring generation, 1926)

Tree species	Number of tips	Number of moths	Number of parasites	Approximate percentage of parasitism
Western yellow pine.....	3,500	3,147	346	10
Jack pine.....	800	168	55	25
Norway pine.....	100	48	13	21
Scotch pine.....	600	161	72	31

Table 3 gives a similar comparison of the 1926 rearing of spring-generation parasites. It will be noted that the percentage of parasitism was lower than in 1925 but that the excess in jack pine over that in western yellow pine remains in approximately the same ratio. Scotch pine has an even higher percentage of parasitized larvae. It is possible that the higher percentage of parasitization in jack and Scotch pine may explain in part the relative resistance of these trees to tip-moth injury. The reason for the difference in percentage of parasitism in jack, Scotch, and yellow pines is not entirely clear, but it is possible that the explanation may be found in the comparative size of the tips of these trees. The slender twigs and small buds of the jack and Scotch pines provide less effective shelter for the tip-moth larvae than the heavy, large-budded yellow pine, in which they may be burrowed so deeply that the small parasites can not reach them. This does not, however, account for the comparatively high percentage of parasitized larvae in Norway pine, inasmuch as this species has fairly heavy tips. It should be noted, however, in this connection that the data for Norway pine are based upon records from only 100 tips, whereas 600 tips was the smallest number of other species used. Thus the probable observational error in the case of Norway pine is somewhat higher than with the other species. A larger series of observations might change the percentage of parasitized larvae somewhat.

In addition to the parasitic insects that are present in the plantations, there are other agencies that tend to reduce the tip-moth numbers. One species of Cleridae, *Hydnocera pubescens* Lec., has been reared from tip-moth material, and it is possible that still other predacious beetles prey upon the tip moth. Ants have been observed to pick up the full-grown tip-moth larvae that have dropped to the ground for cocooning and carry them away to their nest, presumably for food. Spiders are also known to feed upon the adult moths and upon larvae that have accidentally fallen into the spider webs.

Arboreal birds are finding their way into the plantations, and some of them are doubtless finding in the tip moth a source of food. In fact, it is not at all uncommon to find injured tips apparently opened by birds and the larvae gone, although no bird has been observed in the act of opening these tips. Birds may become increasingly important as predators.

Work on this problem has not progressed far enough to warrant the recommendation of control measures. A number of promising leads are being followed, however, and it is hoped that effective and economical means of checking this pest may be developed.

#### SUMMARY

The pine tip moth, *Rhyacionia frustrana bushnelli*, is one of the most injurious pests present in the Bessey plantations in Nebraska.

It is apparently a geographic variety of the Nantucket pine tip moth, a well known but seldom injurious insect in the eastern part of the United States and Canada.

In Nebraska the pest has been present in large numbers since 1909 and the outbreak shows no sign of abating.

This pest probably was introduced in forest-pulled stock shipped in during the early years of the plantation.

This insect mines the tips of seedling and sapling pines and causes deformity, loss in height growth, and occasionally death of the trees attacked.

The western form winters as pupae in cocoons spun in the ground, whereas the eastern form winters in the infested tips of the trees.

The tip moth has two generations a year. The first flight of moths occurs in April and May and the second late in June and July. The eggs are laid singly on the trees near new growth and the larvae tunnel the buds and young tips. The pupae of the first generation, unlike those of the second, are found in the infested tips.

The severity of infestation varies with species and size of the pine.

A number of parasites are attacking the tip moth, but they do not appear able to hold it in check. It is possible that the most effective parasitic species are not present.

The parasites appear to be more effective in jack and Scotch pine than in western yellow pine, possibly because the larger buds and tips of the yellow pine provide better protection for the larvae.

No satisfactory control measures for this pest have been developed as yet. Further investigations, however, are under way, and it is hoped that some practical means of control may soon be developed.



# EFFECT OF HYDROGEN-ION CONCENTRATION ON THE ABSORPTION OF PHOSPHORUS AND POTASSIUM BY WHEAT SEEDLINGS<sup>1</sup>

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## INTRODUCTION

Absorption by plants is controlled by two groups of factors, one affecting the availability of the material to be absorbed and the other the absorbing power of the plants. Although the hydrogen-ion concentration of the medium may affect the solubility of the plant-food constituents, its effect on the absorbing power of the plant only was considered in the investigation here reported. Its effect on growth received no attention.

## METHODS AND MATERIALS

The experiments were conducted in the laboratory with water cultures of wheat seedlings prepared as previously described (5).<sup>2</sup> Purple-straw wheat was germinated on floating perforated aluminum disks and grown, supported by paraffined paper, in glasses of 225 c. c. capacity. In order to prevent absorption of plant food from ungerminated seed before the seedlings were transferred to the glasses, care was taken to keep the seeds on the disks from coming in contact with one another and to prevent their dropping into the germinating pans (3).

The seedlings were removed from the disks about two days after germination became visible and were grown for a day or two in tap water before being transferred to the experimental solutions, in order to stimulate root development. Thirty seedlings were grown in each glass. The germinating pans and the water cultures were kept in a large pan surrounded by a moat of oil to prevent injury by ants or roaches.

The seedlings were grown in the experimental solutions for from one to two weeks. Although the solutions were not balanced, the plants looked normal and healthy throughout the experiments, as their needs for growth were adequately supplied by the food materials stored in the mother seeds. Thus it was possible to study the influence of the reaction of the medium on absorption of mineral plant food by the seedlings, without the disturbing influence of previous absorption (7).

Chemically pure materials were used for the experimental solutions. The concentrations were made relatively high in order to maintain the buffer properties of the solutions, eliminate deficiency in the plant food constituents as a possible limiting factor, and reduce the relative

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<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 345.

differences in concentration resulting from absorption by the growing plants. The general character of the results was preserved also in solutions of low concentrations, when proper conditions were maintained.

Absorption was measured by analyzing all of the plant material obtained from each culture. The ash was dissolved and divided into two equal parts, one of which was used for the determination of phosphorus and the other for the determination of potassium. Absolute absorption was measured by comparison with control cultures of seedlings grown in distilled water. Most of the experiments were carried out in triplicate. The hydrogen-ion concentrations were determined colorimetrically.

#### EFFECT OF INITIAL HYDROGEN-ION CONCENTRATION ON CHANGES IN REACTION PRODUCED BY GROWING SEEDLINGS IN SINGLE-SALT SOLUTIONS

Seedlings were grown in a number of potassium salt solutions and in a sodium chloride solution which contained 750 parts per million of potassium chloride and equivalents of the other salts. The experiment was conducted in two series. In the first the initial hydrogen-ion concentrations ranged from 5.0 to 5.3 and in the other from 6.3 to 6.7. Hydroxides and acids corresponding to the basic and acid radicals of the salts were used to obtain the desired PH values.

TABLE 1.—Changes in hydrogen-ion concentrations produced in solutions of potassium salts and in a solution of sodium chloride by growing wheat seedlings

Salt	PH of solutions having initial PH of 5.0 <sup>a</sup> after—				PH of solutions having initial PH of 6.7 <sup>b</sup> after—			
	1 day	2 days	4 days	8 days	1 day	2 days	4 days	8 days
Potassium chloride.....	3.7	3.5	3.5	3.6	3.9	3.7	3.5	3.8
Potassium nitrate.....	3.9	4.1	4.6	5.5	4.2	4.4	4.9	6.5
Potassium sulphate.....	3.5	3.5	3.4	3.4	3.7	3.5	3.5	3.7
Potassium phosphate.....	3.9	3.5	3.4	3.4	6.2	5.9	5.6	5.6
Potassium acetate.....	5.0	5.0	6.9	7.3	5.8	6.0	6.4	7.1
Sodium chloride.....	4.7	4.7	5.3	6.3	4.9	5.3	6.3	6.8

<sup>a</sup> The initial PH of the sodium chloride solution was 5.3.

<sup>b</sup> The initial PH of the potassium acetate solution was 6.3.

The growing seedlings increased the acidity of all experimental solutions except that of potassium acetate (Table 1). This is in agreement with the results of Breazeale and LeClerc (1), who found titrable acidity in potassium sulphate solutions in which wheat seedlings had been grown, and also with those of Hoagland (?), who found that solutions of three potassium salts increased in acidity under the influence of growing barley seedlings, attaining final hydrogen-ion concentrations of 3.4 to 4.0.

Preferential absorption thus seems to favor the bases, at least in the early stages of growth. The fact that sodium, which is not an essential element of plant growth, was also at first taken up in excess over the acid radical suggests that preferential absorption for bases is chemical in nature. The subsequent decrease in acidity in the sodium chloride solutions indicates the migration of sodium back to the solutions. According to Wilfarth, Römer, and Wim-

mer (14), even essential elements of plant food migrate back to the soil at certain stages of growth.

The potassium nitrate solutions developed less acidity than the other potassium salts because of preferential absorption of the nitrate radical. In the potassium acetate solutions the acid radical was evidently attacked by microorganisms (5).

The initial hydrogen-ion concentration affected the results only in the potassium phosphate solutions. The more acid solutions of this salt behaved like those of the other salts, but the less acid solutions developed only slight acidity.

The experiment was therefore repeated, potassium phosphate and potassium sulphate solutions of several initial hydrogen-ion concentrations being used. The solutions were twice as concentrated as those used before.

TABLE 2.—*Changes in hydrogen-ion concentration produced by growing wheat seedlings in potassium phosphate and potassium sulphate solutions of different initial hydrogen-ion concentrations*

Initial PH	PH of potassium phosphate solutions after—				PH of potassium sulphate solutions after—			
	1 day	2 days	3 days	4 days	1 day	2 days	3 days	4 days
3.6-----	3.3	3.3	3.3	3.3	3.4	3.4	3.3	3.3
4.0-----	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4
5.0-----	3.8	3.7	3.7	3.6	3.7	3.6	3.6	3.6
6.0-----	5.7	5.7	5.6	5.6	3.7	3.6	3.6	3.6
7.0-----	6.8	6.8	6.8	6.8	3.7	3.7	3.6	3.6

The results (Table 2) agree with those of the first experiment. All potassium phosphate solutions with initial hydrogen-ion concentrations up to 5.0 behaved like the potassium sulphate solutions. Those with initial PH values of 6.0 and 7.0, however, developed only slight acidity.

The final hydrogen-ion concentrations in these experiments never attained a value lower than 3.3.<sup>3</sup>

#### ABSORPTION OF PHOSPHORUS AND POTASSIUM FROM SOLUTIONS OF DIFFERENT HYDROGEN-ION CONCENTRATIONS

The effect of the hydrogen-ion concentration of the medium on the absorption of phosphorous and potassium by seedlings was next studied.

Wheat seedlings were grown for two weeks in solutions containing 1,500 parts per million of monopotassium phosphate which had an original hydrogen-ion concentration of 4.9. The same concentration was used in all subsequent experiments unless otherwise stated. The higher and lower PH values were obtained by using potassium hydroxide and phosphoric acid, respectively.

Normal potassium hydroxide solution was added at the rate of 2.8 and 6.6 c. c. per liter of solution to obtain PH 6.0 and 7.0. The quantity of phosphoric acid required to produce the lower values was relatively insignificant.

<sup>3</sup>On the specific acidity scale, in which PH 7=1, PH 3.3 is 5,000.

The seedlings grown in the solutions which increased markedly in acidity absorbed more phosphorus and less potassium than those grown in the solutions which changed but slightly in hydrogen-ion concentration (Table 3). This is just the reverse of what would be expected in view of the fact that the changes in hydrogen-ion concentration were presumably due to preferential absorption. This seeming abnormality, however, is readily explained by a closer study of the results. The seedlings absorbed relatively more potassium than phosphorus from all solutions, regardless of the initial or final hydrogen-ion concentration (columns 8 and 9, Table 3). This preferential absorption of potassium was responsible for the increased acidity in the solutions with the initial  $P_H$  5.0 and lower, as they contained only monopotassium phosphate, which has but slight buffer properties. The solutions with the higher initial  $P_H$  values, containing mixtures of mono- and di-potassium phosphate, had much higher buffer properties and therefore changed but slightly in hydrogen-ion concentration.

TABLE 3.—*Absorption of phosphorus and potassium by wheat seedlings from potassium phosphate solutions of different initial hydrogen-ion concentrations*

PH	Total quantity				Quantity absorbed		Ratio of potassium to phosphorus absorbed	Ratio of potassium to phosphorus in solution	Phosphorus (P) equivalent of absorbed potassium	Excess of phosphorus (P) in solution	Excess of phosphorus in solution in terms of $H_3PO_4$	$H_3PO_4$ required to produce final PH value in 220 cc. of solution	$H_3PO_4$ in excess over that required to produce final PH value
Initial	Final	$P_2O_5^a$	$K_2O^a$	$P_2O_5$	$K_2O$								
		Mgm.	Mgm.	Mgm.	Mgm.			Mgm.	Mgm.	Mgm.	Mgm.	Mgm.	Mgm.
3.6.....	3.3	19.3	14.2	14.9	11.8	151:100	126:100	7.8	1.3	4.1	1.8	2.3	2.3
4.0.....	3.4	18.0	14.2	13.6	11.8	166:100	126:100	7.8	1.9	6.0	2.2	3.8	3.8
5.0.....	3.6	17.8	13.9	13.4	11.5	161:100	126:100	7.5	1.6	5.1	1.7	3.4	3.4
6.0.....	5.6	13.4	16.3	9.0	13.9	295:100	<sup>b</sup> 158:100	7.3	3.4	10.8	0.01	10.8	10.8
7.0.....	6.8	12.6	16.0	8.2	13.6	314:100	<sup>b</sup> 201:100	5.6	2.0	6.3	0.0004	6.3	6.3
Controls.....		4.4	2.4										

<sup>a</sup> In 15 seedlings.

<sup>b</sup> Including the potassium added as potassium hydroxide to obtain the required initial  $P_H$  values.

The excess of phosphoric acid over potassium in the residual solutions does not agree with their final reactions (columns 9 to 13, Table 3). This discrepancy in the solutions with the initial  $P_H$  6.0 and 7.0 may be accounted for by their high buffer properties, but in the more acid solutions it can not be explained so easily. It is possible that the dissociation of the free phosphoric acid is decreased by the presence in minute quantities of organic matter, or that the acid is neutralized by small quantities of organic and inorganic bases excreted by the plant roots. In either case the excretions would be so slight that they could not be detected by analysis in aliquots of 15 seedlings.

The experiment was repeated in two series. In one the desired hydrogen-ion concentrations were obtained by the addition of potassium hydroxide and phosphoric acid, in the other by adding hydrochloric acid and sodium hydroxide. The seedlings were grown in the solutions for seven days. The results are given in Table 4.

TABLE 4.—*Phosphorus and potassium absorbed by wheat seedlings from potassium phosphate solutions of different initial hydrogen-ion concentrations obtained with varied reagents*

Initial PH of solution	From solutions treated with phosphoric acid and potassium hydroxide		From solutions treated with hydrochloric acid and sodium hydroxide	
	P <sub>2</sub> O <sub>5</sub> <sup>a</sup>	K <sub>2</sub> O <sup>a</sup>	P <sub>2</sub> O <sub>5</sub> <sup>a</sup>	K <sub>2</sub> O <sup>a</sup>
	Mgm.	Mgm.	Mgm.	Mgm.
3.6.....	15.3	13.7	13.1	12.5
4.0.....	15.7	14.8	13.9	13.2
5.0.....	14.9	14.2	14.3	13.2
6.0.....	12.1	15.4	11.9	15.0
7.0.....	10.9	13.9	11.7	13.4

<sup>a</sup> In 15 seedlings.

The analytical results of the two experiments (Tables 3 and 4) are not comparable, because of the difference in duration and environmental factors, which are never identical in experiments conducted at different times.

The use of sodium hydroxide and hydrochloric acid instead of potassium hydroxide and phosphoric acid had no effect on the results. The influence of the initial PH of the solutions on the absorption of potassium, shown in the preceding experiment, was not corroborated. In both series, however, the absorption of phosphorus is distinctly higher from the solutions with the initial PH 5.0 and lower from those with the initial PH 6.0 and 7.0. The line between these two sets of solutions is just as sharp as in the preceding experiment.

A similar tendency has been recorded by Hoagland (6), who used older plants and a complete nutrient solution. His solutions, however, contained enough calcium to precipitate most of their phosphorus at the lower hydrogen-ion concentrations. Similarly, Némec and Gračanin (9), who grew rye seedlings in a mixture of soil and sand with the addition of various phosphate fertilizers, found that more phosphorus was absorbed by the seedlings when the reaction of the medium was PH 5.0 than when it was 6.2 or 7.1, but here also the difference in absorption may have been due to differences in solubility. In the present experiments, however, it is clearly not a question of solubility, as all the potassium phosphates are freely soluble. A definite correlation between the hydrogen-ion concentration of the medium and the absorptive power of the plant is therefore established.

#### EFFECT OF DURATION OF CONTACT OF SEEDLINGS WITH SOLUTIONS ON ABSORPTION

Six sets of cultures, which were discontinued at definite intervals, were used. As the initial hydrogen-ion concentration of 5.0 had proved to be the line of demarcation between the two rates of absorption, only two series of solutions were used, one with a PH value of 4.9 which expresses the original hydrogen-ion concentration of the solutions (1,500 parts per million of monopotassium phosphate in laboratory distilled water), and the other with a PH value of 7.0, obtained by adding potassium hydroxide.

The final reaction was practically the same in each set, showing that the change in reaction occurs during the first 24 hours (Table

5). The absorption of phosphorus and potassium increased gradually with the duration of the experiment. Absorption of potassium was greater in the neutral (PH 7.0) series, with one exception. Absorption of phosphorus was greater in every set of the acid (PH 4.9) series.

TABLE 5.—Effect of duration of contact of wheat seedlings with potassium phosphate solutions of different initial hydrogen-ion concentrations on their absorption of phosphorus and potassium

Duration of contact	From initial PH 4.9 solutions			From initial PH 7.0 solutions		
	Final PH	P <sub>2</sub> O <sub>5</sub> <sup>a</sup>	K <sub>2</sub> O <sup>a</sup>	Final PH	P <sub>2</sub> O <sub>5</sub> <sup>a</sup>	K <sub>2</sub> O <sup>a</sup>
Days		Mgm.	Mgm.		Mgm.	Mgm.
1.....	3.6	6.9	7.1	6.9	6.6	7.6
2.....	3.6	9.0	9.2	6.9	8.1	11.2
4.....	3.5	12.0	11.6	6.9	9.1	11.2
6.....	3.5	13.7	11.7	6.9	11.5	14.9
10.....	3.5	15.3	15.9	6.9	11.4	16.5
14.....	3.7	18.3	17.7	6.9	15.6	19.7

<sup>a</sup> In 15 seedlings.

Within the period of these experiments, therefore, the duration of contact of the seedlings with the solutions does not affect the differences in the rate of absorption of phosphorus caused by variations in the hydrogen-ion concentration of the medium.

#### EFFECT OF AGE OF SEEDLINGS ON ABSORPTION

Five lots of seeds were placed for germination at intervals of 2 to 3 days. After 5 days in the germinating pans the seedlings were transferred to glasses and grown in tap water until the last lot was ready for use. The seedlings, therefore, differed in their stages of advancement by periods of 2 days and in one case by 3 days. These differences in age were discernible throughout the experiment. All seedlings were grown in the experimental solutions for 6 days.

Both the absorption and final reaction of the solutions were distinctly affected by the age of the seedlings (Table 6). In the acid series the acidity produced by the growing seedlings decreased as their age at the time of transfer to the solutions increased. In the neutral series a similar tendency was presumably obscured by the buffer properties of the solutions. The absorption of potassium and phosphorus decreased also in both series as the initial age of the seedlings increased, the rate of absorption being highest at the lowest starting age. The 5-day seedlings absorbed four times as much potassium and four to five times as much phosphorus as the 14-day seedlings. This impairment of absorbing power with age may be due to the fact that there was no absorption during growth in a medium devoid of mineral plant food. It is also possible that the rate of absorption decreases as the seedlings advance in age, regardless of the presence or absence of plant food in the medium.

TABLE 6.—*Effect of age of wheat seedlings on absorption of phosphorus and potassium from potassium phosphate solutions of different initial hydrogen-ion concentrations*

Age of seedlings <sup>a</sup>	From initial PH 4.9 solutions			From initial PH 7.0 solutions		
	Final PH	P <sub>2</sub> O <sub>5</sub> <sup>b</sup>	K <sub>2</sub> O <sup>b</sup>	Final PH	P <sub>2</sub> O <sub>5</sub> <sup>b</sup>	K <sub>2</sub> O <sup>b</sup>
		Mgm.	Mgm.		Mgm.	Mgm.
14.....	4.1	8.0	6.15	7.1	7.3	5.59
12.....	4.0	9.8	8.01	7.1	7.6	7.31
10.....	3.8	11.4	9.39	7.0	9.9	10.50
7.....	3.3	14.4	14.00	6.9	10.1	13.80
5.....	3.3	21.5	17.90	6.9	14.0	16.10
Controls.....		5.0	2.28		5.0	2.28

<sup>a</sup> Count started with day the seed was set to germinate.<sup>b</sup> In 15 seedlings.

The relative absorption of potassium was irregular, whereas that of phosphorus was consistent in both series. The differences in the absorption of phosphorus caused by the hydrogen-ion concentration of the medium, however, decreased as the starting age increased. The difference for the starting age of 14 days was about 9 per cent and that for the age of 5 days was about 30 per cent.

Total absorption, relative absorption, and the final reaction of the medium are correlated at least in a general way. It is not unlikely, therefore, that all are conditioned by the same factor.

#### EFFECT OF CONCENTRATION ON ABSORPTION

Solutions of relatively high concentrations were used in these experiments for reasons stated at the outset. As normal soil solutions rarely attain such concentrations, however, it was necessary to determine the effect of concentration on the differences in absorption. Accordingly, six sets of cultures were grown for 9 days in potassium phosphate solutions ranging in concentration from 50 to 3,000 parts per million.

As was to be expected, the buffer properties of the solutions in the neutral series decreased gradually in the lower concentrations, beginning with that of 500 parts per million (Table 7). The apparent exception shown by the solution of the lowest concentration was evidently due to the total removal of the potassium phosphate by the plants, PH 5.4 being essentially the hydrogen-ion concentration of distilled water which has come into equilibrium with the carbon dioxide of the air. The final reaction of the corresponding solution in the PH 4.9 series may be similarly explained, the removal of potassium phosphate being not quite complete in this case.

TABLE 7.—*Effect of concentration on absorption of phosphorus and potassium by wheat seedlings from potassium phosphate solutions of different initial hydrogen-ion concentrations*

Concentration of solutions <sup>a</sup>	From initial PH 4.9 solutions			From initial PH 7.0 solutions		
	Final PH	P <sub>2</sub> O <sub>5</sub> <sup>b</sup>	K <sub>2</sub> O <sup>b</sup>	Final PH	P <sub>2</sub> O <sub>5</sub> <sup>b</sup>	K <sub>2</sub> O <sup>b</sup>
		Mgm.	Mgm.		Mgm.	Mgm.
3,000.....	4.0	19.8	16.6	6.9	13.5	15.6
1,500.....	3.5	18.8	15.7	6.9	11.8	14.5
500.....	3.3	10.0	9.9	5.9	10.3	15.5
200.....	3.3	7.6	7.7	3.6	8.7	11.6
100.....	3.6	7.2	5.7	3.6	8.1	9.8
50.....	4.1	6.1	4.1	5.4	6.8	5.9
Control.....		4.4	2.7		4.4	2.7

<sup>a</sup> Parts per million.<sup>b</sup> In 15 seedlings.

The relative absorption of phosphorus from the solutions with concentrations of 1,500 and 3,000 parts per million was the same as in the previous experiments, but it deviated markedly from the normal course in the lower concentrations, beginning with that of 500 parts per million. This was probably due to the changes in hydrogen-ion concentration in the solutions of the neutral series, resulting from their decreased buffer properties. The absorption of potassium was higher from all four concentrations below 1,500 parts per million in the neutral series.

The initial hydrogen-ion concentration of the medium had no effect on the relative absorption of phosphorus from concentrations which approach that of the soil solution. However, the soil solution is constantly replenishing its stock of available plant food. Accordingly, the experiment on concentration was repeated, solutions being renewed daily. The seedlings were grown in the solutions for 8 days.

The PH values of the solutions discarded daily show that the power of the seedlings to change the initial hydrogen-ion concentration of the solutions diminished with every daily renewal (Table 8). The main object of renewing the solutions—to prevent large variations from the initial hydrogen-ion concentrations in the neutral series—was thus attained.

TABLE 8.—Changes in reaction produced by growing wheat seedlings in daily renewed potassium phosphate solutions of different concentrations and different initial hydrogen-ion concentrations

Concentration of solution *	PH of initial PH 4.9 solutions after—								PH of initial PH 7.0 solutions after—							
	1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days	1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days
1,500.....	3.6	4.1	4.7	4.7	4.8	4.8	4.8	4.8	6.9	7.0	7.0	7.0	7.0	7.0	7.0	7.0
500.....	3.6	3.8	4.1	4.3	4.5	4.9	5.0	5.0	6.9	6.9	7.0	7.0	7.0	7.0	7.0	7.0
200.....	3.5	3.7	4.0	4.1	5.1	5.1	5.2	5.3	5.1	6.1	6.3	6.3	6.3	6.3	6.4	6.3
100.....	3.4	3.7	4.0	4.1	5.1	5.1	5.2	5.3	3.9	5.4	6.1	6.2	6.2	6.3	6.3	6.3
50.....	3.4	3.6	4.0	4.1	5.2	5.4	5.4	5.4	3.7	5.0	5.5	5.8	5.9	6.1	6.1	6.1

\* Parts per million.

Absorption of phosphorus was greater from the acid than from the neutral solutions, irrespective of their concentrations, when through renewal they simulated the soil solution (Table 9). It is probable, therefore, that this correlation of absorption with the hydrogen-ion concentration of the medium properly modified may also apply to natural conditions of plant growth.

TABLE 9.—Effect of concentration on absorption of phosphorus and potassium by wheat seedlings from potassium phosphate solutions of different initial hydrogen-ion concentrations renewed daily

Concentration of solution *	From PH 4.9 solutions		From PH 7.0 solutions	
	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O
	Mgm.	Mgm.	Mgm.	Mgm.
1,500.....	9.7	9.25	7.0	8.5
500.....	8.5	8.90	6.6	10.0
200.....	7.5	8.13	5.9	9.40
100.....	7.2	8.32	5.9	8.80
50.....	6.8	—	5.5	7.40
Control.....	3.7	1.70	3.7	1.70

\* Parts per million.

## DISTRIBUTION OF ABSORBED CONSTITUENTS AMONG ROOTS, TOPS, AND SEEDS

In ashing the plant material for analysis a blackening, considered to be indicative of high phosphorus content, was observed in the roots of the seedlings grown in the acid solutions. This led to a study of the distribution of the absorbed phosphorus and potassium among tops, roots, and mother seeds. The usual procedure was followed. At the end of the experiment the seedlings were carefully separated into tops, roots, and seeds, and then oven dried, weighed, and ashed. Aliquots of 45 seedlings were used for analysis.

The dry weights of the tops were highest in the acid series and lowest in the controls (Table 10). The dry weights of the roots were highest in the controls, which is in accord with the common observation that a medium poor in plant food may stimulate root development. The weights of the mother seeds were lowest in the controls, but practically identical in the other two series.

TABLE 10.—*Distribution of potassium and phosphorus absorbed by wheat seedlings from potassium phosphate solutions of different initial hydrogen-ion concentrations in tops, roots, and mother seeds*

Part of plant	From initial PH 4.9 solutions			From initial PH 7.0 solutions			From controls <sup>a</sup>		
	Dry weight	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O	Dry weight	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O	Dry weight	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O
	Gm.	Mgm.	Mgm.	Gm.	Mgm.	Mgm.	Gm.	Mgm.	Mgm.
Tops.....	0.50	33.4	35.4	0.44	20.1	30.6	0.38	9.2	6.6
Roots.....	.14	5.8	3.3	.15	6.7	7.0	.17	1.7	.5
Seeds.....	.20	11.3	3.5	.19	9.2	4.8	.16	2.0	.3
Total <sup>b</sup> .....	.84	50.5	42.2	1.78	36.0	42.4	1.73	12.9	7.4

<sup>a</sup> Seedlings grown in distilled water.

<sup>b</sup> In 45 seedlings.

The roots in the neutral series had, contrary to expectation, the highest phosphorus content, but they also had a relatively larger potassium content. It would seem, therefore, that blackening of ash is an indication of an excess of phosphorus over bases rather than of high phosphorus content in an absolute sense.

The total potassium content of the seedlings in the two series was about the same, but in the distribution of this element the tops of the acid series were more favored. They also contained the entire excess of phosphorus absorbed by the seedlings of this series. This emphasizes the significance of the hydrogen-ion concentration of the medium as a factor in the physiological availability of phosphorus to plants brought out in these experiments.

## DISCUSSION

The behavior of seedlings with respect to absorption of phosphorus is analogous to that of certain inorganic gels which, according to Starkey and Gordon (12), also absorb more phosphorus from potassium phosphate solutions as their hydrogen-ion concentrations increase. Here, however, preferential absorption was always in favor of the potassium cation, regardless of the rate of absorption

of phosphorus. The phenomena observed can be more plausibly explained on the basis of the isoelectric relations of the colloidal components of the living cell, particularly the proteins. Robbins (11) offered this explanation to account for the differences in absorption of water and dyes by potato tissue on immersion in solutions of different hydrogen-ion concentrations.

According to Pearsall and Ewing (10), the isoelectric point of wheat lies between  $\text{pH}$  3.3 and 4.5. The more acid limit happens to be identical with the lowest limits of acidity produced by the wheat seedlings in these experiments. Csonka, Murphy, and Jones (2) found that the isoelectric point of proteins lies between higher  $\text{pH}$  values, approaching more closely the hydrogen-ion concentration the cell sap in 2-week wheat seedlings, which is about  $\text{pH}$  6.0, according to Hurd (8). However, it is safe to assume that the isoelectric points of proteins of the living cell are not identical with those of isolated proteins which have been subjected to various treatments in the process of extraction and purification. It is further to be assumed that the hydrogen-ion concentration of the cell content is not made identical with that of the medium, but only modified by it. The preferential absorption of potassium is accordingly explainable by the supposition that as long as the acidity of the medium does not increase beyond  $\text{pH}$  3.3 most of the colloids in the wheat seedlings are on the electro-negative side of their isoelectric points and therefore combine with basic radicals in excess over acid radicals. However, as the acidity of the solutions increases because of the preferential absorption of potassium, some colloids pass to the electro-positive side of their isoelectric points and an increased absorption of phosphorus follows. When the acidity of the medium increases momentarily beyond  $\text{pH}$  3.3 the absorption of phosphorus increases rapidly and this value is restored, the preferential absorption for potassium being thus maintained.

The assumption that there is a relatively wide range in the isoelectric point of the ampholytes of growing plants explains not only the absorption phenomena observed in the experiments here reported but also the general mechanism of absorption of the living cell. This diversity of isoelectric points, allowing the occurrence of both electro-positive and electro-negative ampholytes within certain limits of hydrogen-ion concentration, makes possible the simultaneous absorption of cations and anions.<sup>4</sup>

Although it is realized that it is not always safe to apply to field conditions conclusions drawn from laboratory experiments, the facts brought out in these experiments may have a practical bearing on plant production. For example, the high absorptive power of young seedlings would suggest caution in the practice of thinning, in order not to remove too much available plant food from the immediate vicinity of the remaining plants (4). The beneficial effect of transplanting young seedlings may be explained on the same basis. When thick their intensive feeding exhausts the soil, so that they are benefited by being transplanted into fresh soil. They also bring to their permanent ground a store of plant food derived from other sources, which in a way is equivalent to an application of fertilizers.

<sup>4</sup>Stearn's (13) hypothesis that a mixture of ampholytes acts as an individual with one isoelectric point lying somewhere between the  $\text{pH}$  values of the components, would hardly apply to the proteins of living organisms, as these are presumably deposited in separate layers or in different cells and would be expected to react individually.

The fact that the physiological availability of phosphorus to plants depends, among other factors, upon the hydrogen-ion concentration of the medium, emphasizes anew the importance of soil reaction. This may be one of the reasons why some plants appear to prefer an acid medium. It also suggests that the beneficial effect of the application of potassium fertilizers may be due partly to the fact that they increase the acidity in the immediate vicinity of the growing plants. The increased absorption of potassium, then, results indirectly in an increased absorption of phosphorus and perhaps of the other acid-forming elements of plant food also.

#### SUMMARY

Wheat seedlings were grown in potassium phosphate solutions of different initial hydrogen-ion concentrations.

Relatively more potassium than phosphorus was absorbed by the seedlings, irrespective of the initial hydrogen-ion concentration of the solution. In the solutions with initial hydrogen-ion concentrations of 5.0 and lower this preferential absorption of potassium resulted in increased acidity. In solutions with initial hydrogen-ion concentrations of 6.0 and 7.0 the increase in acidity was but slight, owing to the buffer properties of the solutions.

More phosphorus was absorbed by the seedlings from the solutions with initial  $P_H$  values of 5.0 and lower than from those with  $P_H$  values of 6.0 and 7.0. As all potassium salts of phosphoric acid are soluble, this tends to show that the physiological availability of phosphorus depends upon the hydrogen-ion concentration of the medium.

The general character of the results was not affected by the duration of the experiments nor by the age of the seedlings. Neither was it affected by the concentration of the solution, provided the differences in initial reactions in the solutions of the lower concentrations were maintained by daily renewal.

The excess of phosphorus absorbed from the acid solutions was found in the tops of the seedlings. The tops also had a higher potassium content than those from the neutral solutions. The roots from the neutral solutions contained more phosphorus and almost twice as much potassium as those from the corresponding acid solutions.

The power of the seedlings to absorb phosphorus and potassium decreased as they advanced in age.

The absorption phenomena observed in these experiments, as well as the absorption of cations and anions by living cells in general, are explained by the assumption that there is a relatively wide range in the isoelectric points of individual protoplasmic ampholytes.

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# NOTES ON THE LIFE HISTORY OF THE BUD MOTH, *SPILONOTA OCELLANA* D. & S.<sup>1</sup>

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## INTRODUCTION

A detailed study of the life history and habits of the eye-spotted bud moth, *Spilonota ocellana* D. & S., was started in 1917 and continued for five years. The investigations were conducted in an outdoor insectary at Arendtsville, Pa., and frequent trips were made to near-by orchards to correlate insectary and orchard conditions. Synonymy, food plants, distribution, and bibliography have purposely been omitted, for these have been adequately discussed by Porter,<sup>2</sup> in his bulletin "The Bud Moth." The present paper contributes life history studies with special reference to conditions in southern Pennsylvania.

## NATURE OF THE INJURY

The injury caused by the larvae of the bud moth in Pennsylvania may be described under the following headings: (1) Early injury to buds, foliage, and developing fruit by the partly grown, overwintering larvae; and (2) late injury to mature fruit by larvae which hibernate the following winter.

The partly grown, overwintering larvae become active early in the spring and burrow into the opening flower buds (fig. 1, D), feeding upon the blossoms and thereby lessening the set of fruit. As the leaves unfold, the larvae tie them together with silk and feed within the clusters thus made. Newly formed apples are frequently inclosed in such clusters (fig. 1, C) and are chewed by the larvae, causing them to drop or to become disfigured when they mature. The fruit stems are sometimes gnawed, which weakens them and causes the small fruit to drop. Often the larvae bore into the terminal shoots and during some years considerable injury results. Sanders and Dustan (1919)<sup>3</sup> report that the injury to blossom clusters in Nova Scotia in 1918 was 35.8 per cent and that in some cases the set of fruit was reduced as much as 79.9 per cent. The size of the fruit from injured clusters was also considerably smaller. Although the percentage of injury in Pennsylvania has not been as serious as this, it is evident that this insect can, at times, cause considerable loss.

Late in the summer the young larvae bite into the fruit, making small scars. Such injury is most common where a leaf is in contact with a fruit or where two fruits hang together. Plate 1, H, shows a mature fruit that has been injured by the feeding of a bud-moth

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<sup>2</sup> PORTER, B. A. THE BUD-MOTH. U. S. Dept. Agr. Bul. 1273, 20 p., illus. 1924.

<sup>3</sup> SANDERS, G. E., and DUSTAN, A. G. THE APPLE BUD-MOTHS AND THEIR CONTROL IN NOVA SCOTIA. Canada Dept. Agr. Ent. Branch Bul. 16: 5-30, illus. 1919.

larva. The discolored area also shows the position of the leaf beneath which the larva was feeding at the time the injury was produced. The most serious injury in Pennsylvania is of this type. J. R. Eyer, formerly of this station, found 14.7 per cent of fruit on sprayed trees injured by bud-moth larvae during 1917. In 1918, 5.1

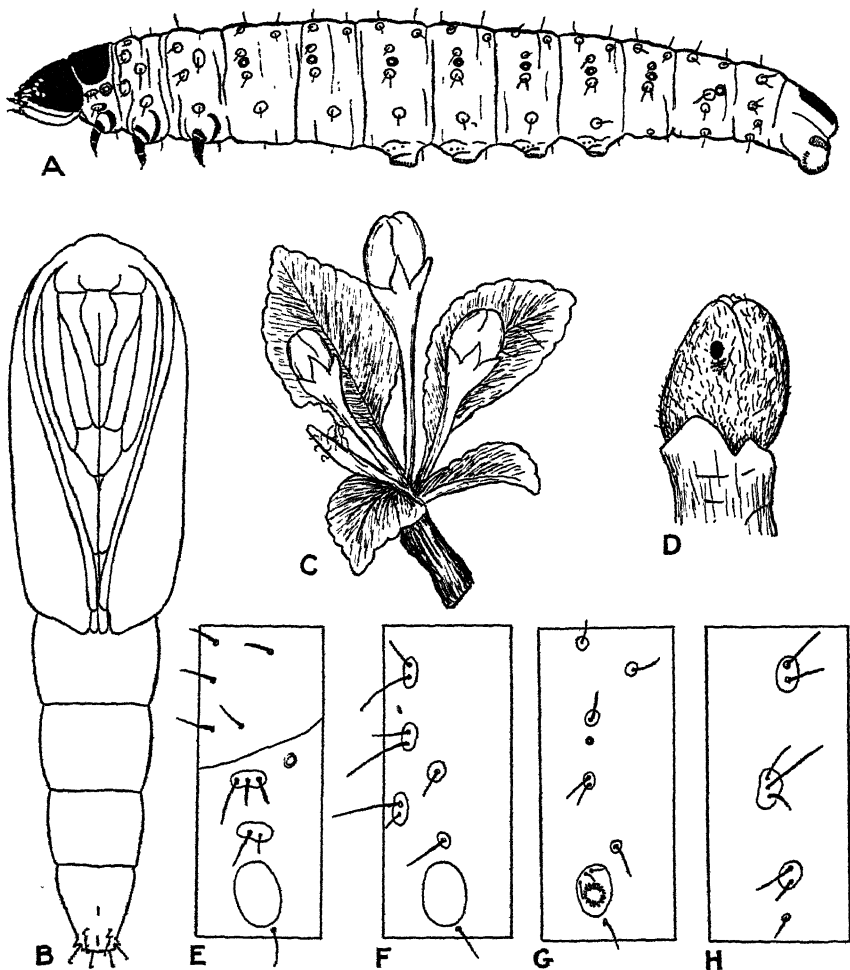
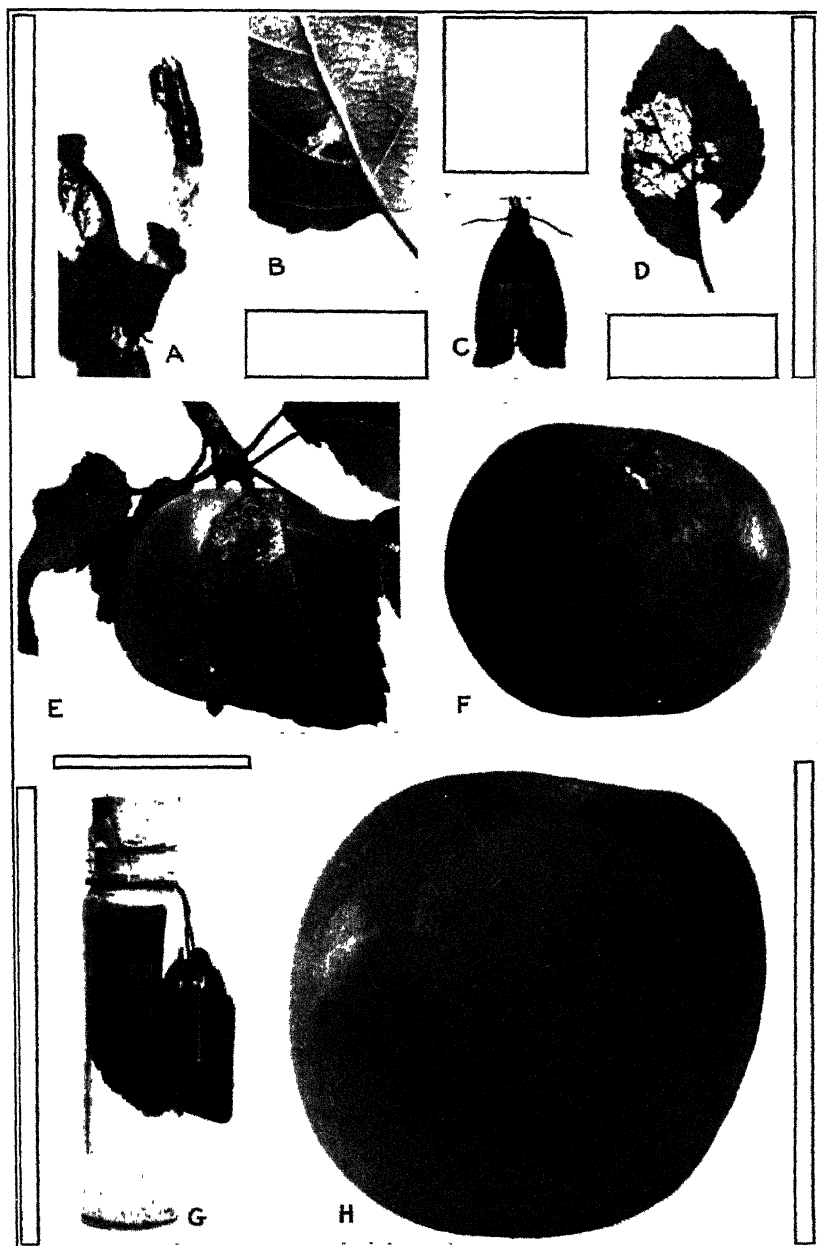


FIG. 1—A, larva of bud moth; B, pupa of bud moth; C, feeding case and leaf cluster; D, bud-moth injury on apple bud; E, prothoracic segment of larva; F, mesothoracic segment of larva; G, third abdominal segment of larva; H, ninth abdominal segment of larva

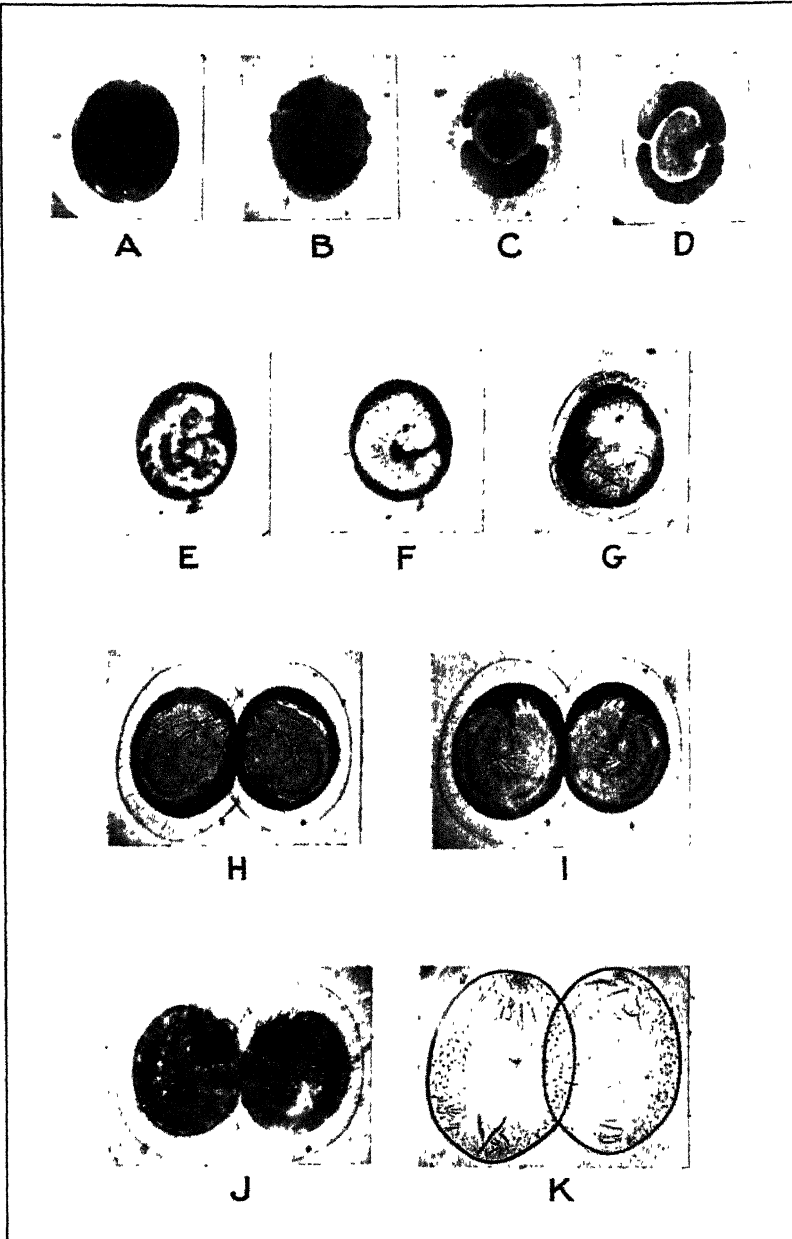
per cent, and in 1919, 10 per cent of the fruit on unsprayed trees were injured by this species.

#### THE ADULTS

The adults (pl. 1, C) were rarely observed flying in orchards and records show that they are seldom attracted to lights or sugar baits. In captivity one can rear them with ease if they are provided with water or sugar solution. This seems necessary in order to keep the adults from dying prematurely.



- A.—Empty pupal case.  
 B.—Position of larva immediately after hatching, under silken tent.  
 C.—Adult of *Spilonota ocellana* D. & S.  
 D.—Feeding case and manner of feeding of larva.  
 E.—Leaf tied to fruit by larva of bud moth.  
 F.—Same as in E with leaf removed.  
 G.—Rearing bottle, with bud-moth pupa on leaf, natural size.  
 H.—Feeding punctures of bud-moth larva, showing discolored area on fruit caused by leaf cutting off the sunlight.



- A.—Egg immediately after oviposition.  
 B.—Egg showing migration of yolk to opposite ends.  
 C.—Yolk gathered at opposite ends of egg, embryo visible in middle.  
 D.—Yolk flowing about embryo.  
 E.—Embryo 51 hours old, showing leg buds.  
 F.—Embryo showing segmentation and legs pressed against thorax.  
 G.—Horizontal revolutions of embryo.  
 H.—Six-day-old embryos after vertical revolution.  
 I.—Advanced stage of H, showing abdomen elongated and curved at the tip.  
 J.—Mature embryo, head fully colored, and yolk consumed.  
 K.—Empty egg shells.

The length of life of the adult moth varies from 5 to 26 days, the average for both males and females being approximately 14 days. A length-of-life record kept in 1919 is shown in Table 1.

TABLE 1.—*Length of life of adult male and female eye-spotted bud moths, 1919*

Males				Females			
Number of adults	Adults issued	Adults died	Length of life (days)	Number of adults	Adults issued	Adults died	Length of life (days)
1.....	June 2	June 18	16	1.....	June 2	June 24	22
1.....	June 3	June 24	21	1.....	June 3	June 24	21
1.....	June 5	June 23	18	1.....	June 4	June 12	8
1.....	June 6	June 16	10	1.....	June 5	June 23	18
1.....	June 6	June 23	17	1.....	June 6	June 16	10
1.....	June 6	June 24	18	1.....	June 6	June 19	13
1.....	June 6	July 2	26	1.....	June 6	June 24	18
1.....	June 7	June 16	9	1.....	June 6	July 2	26
1.....	June 7	June 17	10	1.....	June 7	June 16	9
1.....	June 7	June 18	11	1.....	June 7	June 17	10
1.....	June 7	June 19	12	1.....	June 7	June 24	17
1.....	June 7	June 20	13	1.....	June 8	June 23	15
1.....	June 7	June 21	14	1.....	June 9	June 23	14
1.....	June 8	June 18	10	1.....	June 9	June 24	15
1.....	June 9	June 16	7	1.....	June 9	June 28	19
				1.....	June 11	June 26	15
				1.....	June 12	June 25	13
				1.....	June 12	June 28	16
				1.....	June 13	July 2	19
				1.....	June 13	June 20	5
				1.....	June 13	June 25	10

NOTE.—The average length of life of the 15 adult males was 14.1 days; of the 21 females, 14.9 days.

Copulation takes place soon after the adult emergence, and several days elapse before egg laying commences. In one instance this period lasted for 17 days, but this is apparently abnormal. Table 2 summarizes the preoviposition period of a few females in 1918 and 1919.

TABLE 2.—*Preoviposition period of the eye-spotted bud moth, 1918 and 1919*

Year	Number of females	Date adults emerged	Date first eggs were laid	Length of period (days)
1918.....	1	June 3	June 10	7
1918.....	2	June 5	June 10	5
1918.....	1	June 8	June 25	*17
1919.....	1	June 2	July 5	3
1919.....	1	June 3	July 5	2
1919.....	1	June 4	July 6	2
1919.....	1	June 5	July 7	2
1919.....	2	June 6	July 7	1
1919.....	1	June 6	July 8	2
1919.....	2	June 7	July 9	2
1919.....	1	June 7	July 10	3
1919.....	1	June 8	July 11	3
1919.....	1	June 8	July 12	4
1919.....	2	June 9	July 10	1
1919.....	2	June 9	July 11	2
1919.....	1	June 11	July 19	8
1919.....	2	June 12	July 16	4

\* Abnormal.

NOTE.—The average length of the preoviposition period for the 23 females studied was 3.6 days.

## OVIPOSITION

The egg-laying activities of the moths were observed many times. As the time for oviposition approaches, there are indications of excitement on the part of the female followed by periods of quietness, during which the tip of her abdomen touches the surface upon which she rests. When ready to oviposit the female lifts the tip of her abdomen and spreads her genitalia so as to expose the opening of the oviduct. Almost immediately a glistening white egg appears at the opening of the ovipositor. The tip of the abdomen is then quickly lowered to the surface of the leaf, and an egg is deposited. The abdomen is raised again, and, after half a minute, or possibly longer, a second egg appears. Several eggs are thus laid in succession. In the case of one female observed, 11 eggs were laid in about 30 minutes. In all cases oviposition occurred at night.

The eggs are laid singly or in overlapping patches of from 2 to 20. They are fastened to the leaf with a viscous material which soon hardens on exposure to the air. This cementlike substance does not dissolve when the eggs are preserved in alcohol. The margin of the egg is firmly attached to the leaf while the center bulges slightly to accommodate the yolk and protoplasm.

The number of eggs laid by a single female varies considerably: The smallest number of eggs laid by a single moth was 28 and the greatest number 450. A record of the number of eggs laid by a group of moths in 1920 and 1921 is summarized in Tables 3 and 4.

TABLE 3.—Oviposition and number of eggs laid by the eye-spotted bud moth, 1920\*

June																				July										Number of eggs laid
11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1	2	3	4	5						
♂♂																														

\* ♂ and ♀ at left of chart indicate dates when male and female moths emerged and those at right of chart indicate dates when the males and females died. No observation was made on days marked (?). The eggs were counted the following day.

NOTE.—There were 21 pairs of adults under observation, and the total number of eggs laid was 2,953.

TABLE 4.—Oviposition and number of eggs laid by the eye-spotted bud moth, 1921 \*

June																												July			Num- ber of eggs laid		
2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1	2		3	
♂	0	0	110	49	35	31	15	20	21	19	8	13 (?)	27	8	8	0	11	0	0	11	♀											386	
♀	0	128	44	39	30	22	13	31	19	23	19	23 (?)	26	15	10	8	3	0	0	20	♀											450	
♂		0	95	43	26	29	14	26	♀																							233	
♂		0	0	0	0	0	0	0	0	0	♀	0	0	29	0	0	0	♂	0													29	
♀		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	(?)	18		0	0	♀							28	
♀		0	0	0	0	0	0	0	0	0	0	6	13 (?)	14	5	9	4	0	0	0	♀											188	
♀		0	0	0	0	0	0	0	0	0	0	9	3 (?)	15	♀																	156	
♀		0	0	0	0	0	0	0	0	0	0	3	0 (?)	26	11	18	14	0	0	0	15		5	0	0	0	1	0	0	0	♂ ♀	192	
♀		0	0	0	0	0	0	0	0	0	0	21 (?)	0	0	♀																	181	
♀		0	0	0	0	0	0	0	0	0	0	0	21 (?)	0	0	0	0	♀														159	
♀		0	0	0	0	0	0	79	6	62	13	0	3	0	♀																		163
♀		0	0	0	0	56	0	45	18	0	{ 11	?	45	6	9	6	0	0	0	0	0	♀											196
♀		0	0	0	0	0	0	0	4	82	2	0 (?)	26	0	4	♂	♂	0	0	0	0	0	♀										118
♀		0	0	0	0	0	0	0	0	76	2	0 (?)	113	0	32	♂	0	0	0	(?)	31		5	0	0	0	♀						279
♀		0	0	0	0	0	0	0	0	53	30	0	30	32	10	0	17	0	0	0	(?)	15		0	0	♀							187
♀		0	0	0	0	0	0	0	37	88	0	47	33	19	16	15	8	0	0	0	♀	5											268
♀		0	0	0	0	0	0	0	77	13	0	0 (?)	82	0	22	♂	0	0	0	0	♀												194
♀		0	0	0	0	♀	35	46	46	12	0	(?)	71	0	{ 11	0	8	♀															229
♀		0	0	0	0	♀	0	0	0	57	0	0 (?)	95	0	29	0	0	0	0	0	3	♀	3										187
♀		0	0	0	0	♀	0	0	0	0	0	0 (?)	129	0	51	12	0	0	0	0	♂	29	♀										221
♀		0	0	0	0	♀	0	0	0	0	0	0	0	72	0	28	♂	0	0	0	8		0	0	0	1	♀						109

\* ♂ and ♀ at left of chart indicate dates when male and female moths emerged; at the right of chart they indicate dates when male and females died. (?) indicates that no records were made.

NOTE.—The total number of eggs laid by females of 21 pairs of moths was 4,133.

### THE EGG

The egg measures 0.8 mm. in length by 0.6 mm in width (pl. 2, A). It is much flattened or scalelike, oval in shape, and bluntly pointed at one end. This pointedness is due to the pressure of the ovipositor at the time of deposition. Sometimes the egg is drawn out into a fine hairlike point at one end, but this is not characteristic. It is transparent, milky, and granular, and is more granular at the rounded end. Its contents are surrounded by a thin, almost invisible, vitelline membrane. Outside of this is a rather tough, transparent chorion faintly sculptured with polygonal markings.

The incubation period of the eggs varies from 7 to 12 days. In 1918 it lasted from 7 to 9 days, the average being 8.1 days. A record kept in 1919 on a total of 986 eggs showed that the egg stage lasted from 8 to 12 days, the average being 9.1 days. The data covering these observations are shown in Tables 5 and 6.

TABLE 5.—Incubation period of eggs in 1918

Date eggs laid	Date eggs hatched	Incubation period (days)
June 10.....	June 17 to 18.....	7 to 8
June 9 to 13.....	June 17 to 18.....	8 to 9.
June 10.....	June 17.....	7.
June 9.....	June 17 to 18.....	8 to 9.

TABLE 6.—Incubation period of eggs in 1919

Number of eggs	Date eggs laid	Date eggs hatched	Incubation period (days)
95	June 5.....	June 14.....	9
260	June 7.....	June 15 to 17.....	8-10
58	June 8.....	June 16 to 17.....	8-9
174	June 9.....	June 17.....	8
47	June 8 to 9.....	June 16 to 17.....	8
130	June 9 to 10.....	June 17 to 18.....	8
122	June 11.....	June 19 to 21.....	8-10
62	June 12.....	June 20 to 21.....	8-9
34	June 13.....	June 21.....	8
6	June 18.....	June 28.....	10
2	June 19.....	June 27.....	8
20	June 20.....	June 30 to July 1.....	10-11
5	June 22.....	July 3.....	11
1	June 24.....	July 4.....	10
Total.... 986	Average length of incubation period..		9.1

In the course of a few hours after the egg is laid the yolk becomes pale yellow or straw color and many interesting changes take place which can be followed by means of a hand lens or microscope and illumination from transmitted light. The flatness and thinness of the eggs made it possible to observe the grosser embryonic changes. The eggs were obtained by inducing the females to oviposit on microscope slides. These were examined under the low power of a compound microscope, and numerous observations were made at short intervals of all movements and changes within the eggs. Photomicrographs and diagrams were made illustrating the important changes.

Almost immediately after oviposition, distinct pulsating movements of the yolk are noticeable within the egg. These movements are caused by the migration of the yolk cells toward the opposite ends of the egg (pl. 2, B). During the first few hours, before the yolk gathers at the opposite ends, the blastoderm forms at the larger end of the egg and pushes into the center. This can not be observed in the living egg because the yolk obscures the germ band. Furthermore, the comparative thickness of the egg prevents a view of a single layer of cells. If the egg is placed in weak Flemming's fluid, however, the young embryo can be seen before the yolk moves to the ends of the egg. The first changes are very rapid, and it would be necessary to resort to sectioning to reveal the formation of the blastoderm.

In the course of 10 or 12 hours the yolk gathers in two semi-circular-shaped bodies at the opposite ends of the egg, and in the living egg the embryo becomes visible for the first time (pl. 2, C). The embryo at this time is rounded or slightly kidney-shaped. It consists of two portions, an inner densely granular portion and a less granular outer portion.

The yolk soon commences to flow, by irregular pulsations, about the embryo, and at the end of 24 to 48 hours completely surrounds the embryo (pl. 2, D). Division membranes appear in the yolk, giving it a segmented appearance. The outline of the embryo at this time is not as regular as before, due to the segmented condition of the yolk. A slight shrinking of the contents of the egg and the

pulling of the vitelline membrane away from the edge of the chorion likewise becomes visible.

Approximately 54 hours after oviposition the legs appear as three invaginations in the yolk mass (pl. 2, E). The shape of the embryo becomes more pronounced and the contents of the egg shrink distinctly from the edge of the chorion. The segmentation of the embryo is not visible at this time.

About four days after oviposition the embryo becomes segmented, and the legs are well formed and tightly pressed against the under side of the thorax, which at this stage of development is turned toward the outside of the egg (pl. 2, F). The mouth parts commence to form, and the first indication of eyes is represented by two small reddish spots on opposite sides of the head, one lying directly below the other.

Five days after oviposition the embryo makes a revolution in a horizontal plane (pl. 2, G). This is brought about by the elongation of the embryo and the turning of the tip of the abdomen backward. The legs are thus thrown between the thorax and the abdomen. At this time the mouth parts are very distinct, and a dark line appears close to the exterior wall of the embryo, running from the posterior edge of the thorax to about the middle of the abdomen. This line is probably the mid-intestine. The eye spots and segmentation of the embryo are likewise very prominent. The abdomen, due to its new position, lies straight and is not curled at the tip as before.

In a short time, usually less than 24 hours, a second revolution takes place in a vertical direction which brings the legs under the body. The head and thorax are then turned so that one sees them from the dorsal aspect, both eye spots thereby becoming visible (pl. 2, H). The abdomen elongates and becomes curved again at the tip (pl. 2, I). At the same time the yolk supply is rapidly consumed and the contents of the egg shrink noticeably from the edge of the chorion.

On the tenth or eleventh day the embryo becomes mature and the egg is ready to hatch. The head and anterior margin of the prothoracic shield become chitinated. The supply of yolk is entirely consumed and the embryo occupies all the space within the vitelline membrane (pl. 2, J). The embryo finally emerges by cutting a slit through the chorion with its mandibles (pl. 2, K).

#### THE LARVA

The newly hatched larvae are pale yellow in color, but as soon as the first molt occurs they turn deep yellow, and later pinkish brown. It is not until after the second molt that they assume the deep-brown color characteristic of the mature larva (fig. 1, A). The head and prothorax in all instars are shiny black except immediately after a molt, when they are white or pale gray. Setal maps are shown in Figure 1, E, F, G, H.

The bud-moth larvae resemble somewhat the larvae of *Mineola indigenella* and *Sparganothis idaeusalis* which also attack the apple. The former is similar in color but when full grown is larger and has a very much roughened head. The latter is slightly paler, has a light-brown head and prothoracic shield, is much more active than the eye-spotted bud moth, and does not inclose itself in a feeding tube.

In all cases observed the eggs were laid on the upper side of the leaves, and the larvae on hatching were found on this side. This surface is smooth and does not afford the protection of the under, hairy side. Consequently it was not surprising to find that newly hatched larvae in the laboratory, as well as those placed on the leaves in the orchard, soon migrated to the under side of the leaves. Observations made on 793 larvae, with respect to the side of the leaf preferred by them, are represented in Figure 2.

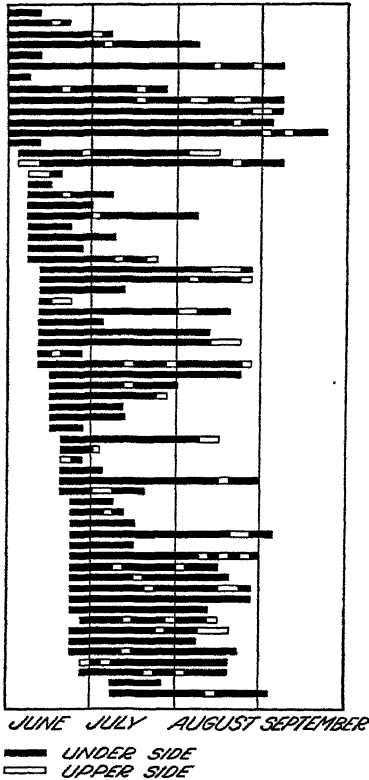


FIG. 2.—Diagram illustrating the position of bud-moth larvae on the under and upper sides of the leaf

#### LARVAL CASES

The newly hatched larvae begin at once to spin silk. At first only a few threads are spun to enable them to maintain their position on the foliage against wind and rain. Later leaves are tied together to form protective shelters inside of which the larvae construct their feeding tubes. These feeding tubes are begun about 24 hours after hatching and are quite compact. At first they are formed on the under side of the leaves usually along the midribs or larger veins. They are white in color and made of silk in which are incorporated plant hairs from the under side of the leaf. They are quite inconspicuous and are readily overlooked. At the end of the first day such cases are about one-sixteenth of an inch long. Two days later they measure about three-sixteenths of an inch (pl. 1, B).

Larvae begin to feed two days after hatching and, as they continue to take food, the frass is ejected from one end of the case and added to the exterior. The outside of the feeding tube soon becomes gray or black as the result of the accumulation of fecula (pl. 1, D). The inner silken layer of the tube, however, remains white or of a slightly creamy color.

The shape of the feeding tubes varies considerably. Some are small tubular shelters almost closed at one end and enlarged at the opposite end. Others are straight tubes open at both ends. Still others are tubular but are covered with a tentlike sheet of silk. The larvae seldom wander from these but sometimes abandon them and construct new ones. The cases are lengthened as the larvae increase their feeding grounds.

## FEEDING HABITS

The larvae are shallow feeders on both foliage and fruit. When feeding on the under surface of a leaf they make holes through the lower epidermis and parenchyma as far as the upper epidermis. When feeding from the upper side they rarely eat through the lower epidermis. Nevertheless they consume a considerable quantity of plant tissue, and as they do not eat single large areas but nibble here and there, it is easy to understand how they become so injurious. A record of the measurements of leaf area eaten by the larvae is given in Table 7.

TABLE 7.—Amount of leaf tissue eaten by eye-spotted bud moth larvae

Number of larvae	Date feeding began	Date feeding ceased	Feeding period (days) <sup>a</sup>	Leaf surface removed (square inches)
1.....	July 3.....	Aug. 7.....	35	1
3.....	July 5.....	July 13 to Nov. 9.....	232	11½
7.....	July 8.....	July 21 to Oct. 28.....	373	18½
2.....	July 10.....	July 26 to Aug. 3.....	39	3½
2.....	July 15.....	Sept. 28 to Oct. 10.....	235	11½
4.....	July 17.....	Aug. 3 to Nov. 20.....	280	10¾
1.....	July 11.....	July 26.....	15	1¾
1.....	July 20.....	Oct. 10.....	82	3¾
1.....	July 26.....	Oct. 22.....	88	4¾
1.....	Aug. 3.....	Oct. 21.....	79	3½
1.....	Aug. 8.....	Oct. 10.....	63	1½
1.....	Aug. 28.....	Oct. 10.....	43	1¾

<sup>a</sup> Figured for the number of larvae feeding on these dates.

The feeding habits of the larvae from midsummer till late fall are very irregular. In most cases they cease feeding entirely and go into a quiescent state. Occasionally quiescent larvae become active and start feeding again. A summary of late summer and fall activities of a number of larvae is given in Table 8.

TABLE 8.—Late summer and fall activities of eye-spotted bud moth larvae

Number of larvae	Date feeding ceased	Date larva entered resting period	Date feeding resumed	Date larva entered resting period
1.....	July 17.....	July 17.....		
1.....	July 18.....	July 21.....		
1.....	July 20.....	July 20.....		
1.....	July 20.....	July 23.....	July 27.....	Aug. 8.....
1.....	July 21.....	July 21.....		
1.....	Aug. 3.....	Aug. 8.....		
1.....	Aug. 15.....	Aug. 26.....		
1.....	Aug. 28.....	Aug. 28.....		
1.....	Sept. 6.....	Sept. 6.....		
1.....	Sept. 26.....	Sept. 26.....		
1.....	Oct. 3.....	Oct. 3.....		
1.....	Oct. 9.....	Nov. 21.....		
1.....	Oct. 10.....	Nov. 25.....		
1.....	Oct. 22.....	Oct. 22.....		
1.....	Oct. 28.....	Nov. 22.....		
2.....	Nov. 9.....	Nov. 9 to Nov. 21.....		
1.....	Nov. 25.....	Nov. 26.....		

Larvae of the eighth, ninth, or tenth instars hibernate in little silken cases on the twigs near the buds. In confinement, many of them burrowed into the cork stoppers of the rearing bottles or into the petioles of leaves, but some formed no hibernacula at all.

## NUMBER OF MOLTS

The number of instars and the molting activities of the larvae were discussed by the writer<sup>3</sup> in 1922. It is very evident that the number varies with the latitude. Sanders (1919)<sup>4</sup> found that there are seven molts in Canada. Porter (1924)<sup>5</sup> records only six for Connecticut. In Pennsylvania there are usually 11 molts, but in some cases 12 molts occur. Overwintering larvae may molt two or three times in the spring after resuming activities. A summary of the number of molts and the average length of the different instars is given in Table 9. It will be noticed that the length of the instar increases with each successive molt.

TABLE 9.—Length of the larval instars of the eye-spotted bud moth, summer, 1918, and spring, 1919

Length of instars (days)	Number of larvae											
	First instar	Second instar	Third instar	Fourth instar	Fifth instar	Sixth instar	Seventh instar	Eighth instar	Ninth instar	Tenth instar	Eleventh instar	Twelfth instar
2	1							5				
3					1							
4	2	1	6		2							
5	20	2	4	1	1		1					
6	29	14	2	1		2						
7	2	12	6	3	1	2						
8	3	13	12	5			2			1		
9	4		2	4	2		1					
10		2	4	4	2		6		1			
11		1		2	4	1	2	2				
12	2	1	6	3	2	2	2	1				
13				5	4			1				
14				5	2	2	2	1				1
15				1	1	2	3	4	2	1		1
16				2	4	1		2	1	1	4	
17				1	2	1	1					
18				1	1		1	1	3	2		
19			1		1	2		1				
20					1	3	1		1			
21				1	1			1				
22						1	1					1
23										2		3
24								2				
25							1		1			
26						1						1
27						1				1		
28							1	1		1		
29											1	
30					1					1		
32											1	
33						1			1	1		1
38											1	
39										1		
42								1	1			
49									1			
Average length of instars (days)	6.1	7.0	7.9	11.3	13.0	16.2	13.3	14.8	21.5	23.2	26.8	22.3
Total number of larvae	63	45	43	39	33	25	24	24	14	10	9	8

## LARVAL MORTALITY

The larvae are very susceptible to injury in confinement and the mortality was unusually high. Possibly similar conditions would be found with larvae under natural conditions. In breeding cages the mortality was higher during the earlier instars. This is well illustrated in Table 10.

<sup>3</sup> FROST, S. W. ECDYSIS IN *TMETOCERA OCELLANA* SCHIFF, Ann. Ent. Soc. Amer. 15: 164-168, illus. 1922.

<sup>4</sup> SANDERS, G. E., and DUSTAN, A. G. Op. cit.

<sup>5</sup> PORTER, B. A. Op. cit.

TABLE 10.—*Mortality of bud-moth larvae during different instars*

Instar	Dead or missing	Instar	Dead or missing
First.....	26	Seventh.....	5
Second.....	49	Eighth.....	2
Third.....	17	Ninth.....	2
Fourth.....	6	Tenth.....	1
Fifth.....	3		
Sixth.....	0	Total.....	111

The number of dead and missing larvae in breeding cages in which approximately 1,000 larvae were handled during 1918 and 1919 was as shown in Table 11.

TABLE 11.—*Occurrence of dead and missing larvae in captivity*

	1918								1919					
	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total	June	July	Aug.	Sept.	Oct.	Total
Dead.....	8	14	1	2	3	4	0	32	18	21	4	1	1	45
Missing.....	10	14	7	2	0	0	0	33	38	24	1	1	2	66
Injured.....	1	0	1	0	1	0	0	3	0	4	1	1	0	6

### THE PUPA

The pupa varies from 7.1 mm. to 8 mm. in length, and is brownish in color (fig. 1, F). It is quite slender and the abdomen tapers gradually toward the posterior end, terminating in a blunt or rounded segment without a cremaster. The dorsal aspect of the abdominal segments bears two rows of spines; a row of strong spines on the anterior margin, and a row of weaker spines across the middle of each segment. The last segment bears about 10 strong spines at the tip, on the dorsal side. There are in addition eight weak hooks, four extending from the dorsal side and four from the ventral surface. The latter are arranged in pairs on each side of the anal opening. The labrum is set off by distinct sutures. The maxillary palpi are about twice as long as the labial palpi and completely inclose them. The femora of the prothoracic legs extend slightly beyond the tips of the maxillary palpi. The prothoracic legs extend half way to the edge, the mesothoracic legs nearly to the edge, and the metathoracic legs slightly beyond the edge of the wing pads. The antennae are comparatively long, reaching nearly to the edge of the wing pads.

Transformation to the pupal stage occurs three or four days after the cocoons are spun. Usually it occurs in the spring. Occasionally, however, a few individuals may transform in late summer when a partial second generation is produced. Pupae are usually found concealed among dried leaves. Before the adults emerge they work themselves part way out of the cocoons. Tables 12 and 13 give the duration of the pupal period from 1918 and 1919.

TABLE 12.—Length of pupal period, 1918

Number of larvae	Date of pupation	Date of emergence of adults	Length of pupal period (days)
1.....	May 20	May 30	10
2.....	May 20	June 1	12
2.....	May 22	June 3	12
1.....	May 28	June 4	7
1.....	May 28	June 5	8
2.....	May 28	June 6	9
1.....	May 28	June 7	10
2.....	May 28	June 10	13

NOTE.—The average length of the pupal period for the 12 larvae studied was 10.5 days.

TABLE 13.—Length of pupal period, 1919

Number of larvae	Date of pupation	Date of emergence of adults	Length of pupal period (days)
1.....	May 29	June 2	4
1.....	May 29	June 4	6
1.....	May 29	June 5	7
2.....	May 29	June 6	8
4.....	May 29	June 7	9
1.....	May 29	June 9	11
3.....	May 30	June 3	4
2.....	May 30	June 5	6
2.....	May 30	June 6	7
1.....	May 30	June 7	8
1.....	May 30	June 16	17
1.....	June 2	June 12	10
1.....	June 2	June 15	13
1.....	June 2	June 17	15
1.....	June 2	June 28	26

NOTE.—The average length of the pupal period for the 23 larvae studied was 9 days.

### NUMBER OF GENERATIONS

Normally there is but one complete generation of the eye-spotted bud moth in Pennsylvania. Observations made in 1918 show a partial second generation. Larvae which hatched from eggs laid about the middle of June continued their feeding until fall. The majority entered hibernation in the eighth and ninth instars and completed their development the following spring, but a few pupated in August and adults issued and laid eggs. During the seasons of 1919, 1920, and 1921, only a single brood occurred. It appears from several years' observations that a single brood is normal.

### PARASITES AND PREDATORS

The parasitic and predacious enemies of the bud moth have been very carefully reviewed by Porter (1924).<sup>6</sup> One species, not mentioned, might be added, namely: *Itoplectis obesus* described by Cushman, 1917.<sup>7</sup> This makes a total of 26 known parasites of the eye-spotted bud moth, 15 of which occur in North America.

<sup>6</sup> PORTER, B. A. Op. cit.

<sup>7</sup> CUSHMAN, R. A. EIGHT NEW SPECIES OF REARED ICHNEUMON-FLIES, WITH NOTES ON SOME OTHER SPECIES. U. S. Natl. Mus. Proc. 53: 457-469. 1917.

A certain mite was frequently found associated with the bud-moth larvae in their feeding cases, but it is questionable whether this mite attacks the bud-moth larvae or simply takes advantage of the shelter offered by the larvae and feeds on excrement or small organisms present.

Several predators have been recorded in literature, namely: A Carabid beetle *Triphleps* sp., a mite *Anystis agilis* Banks, and a mud wasp, *Odynerus Catskillensis* Sauss.

#### SUMMARY

The eye-spotted bud moth is occasionally responsible for severe injury to the fruit and foliage of the apple. It was first reported in this country in 1841, and since that time has been injurious in southern Canada and in at least 24 States in the northern part of the United States.

Injury is caused by the larvae which attack the opening buds, foliage, and newly set fruit in the spring, and the mature fruit in late summer. The injury to mature fruit is the more serious in southern Pennsylvania.

Larvae of the eighth, ninth, and tenth instars hibernate in small hibernacula on twigs near the buds. These emerge early the following spring, and after molting two or three times become mature toward the end of May and pupate within curled leaves. The pupa stage varies from 9 to 10 days. Adults live for an average of 14 days after emergence. There is a preoviposition period of 3 or 4 days. Eggs are laid singly or in small batches of from 2 to 20. They are glued to the leaf with a cementlike substance. The larvae appear about mid-June and feed on fruit and foliage until fall, when they hibernate. Most of their feeding is done on the under side of the leaves. There is high mortality among larvae in breeding cages, particularly during the earlier instars. Normally there is one generation of moths a year.



# RELATION OF MATURITY TO THE NUTRITIVE VALUE OF FIRST, SECOND, AND THIRD CUTTINGS OF IRRIGATED ALFALFA<sup>1</sup>

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## INTRODUCTION

Under conditions of irrigation farming in the Yakima River Valley of Washington three cuttings of alfalfa are usually harvested and produce hay of fine quality. Yields in tons and permanency of stand have been the primary considerations in the production of such hay, only slight emphasis being placed upon nutritive value. Some attention has been given to the stage of maturity (36)<sup>3</sup> in its relationship to yield of the three cuttings. Feeding experiments with lambs over a period of four years have shown the three cuttings of alfalfa harvested in one-half bloom to possess equal values for fattening.

Subsequent digestion and metabolism studies reported in full in this paper supply further information pertaining to the nutritive value of the three cuttings, when each is harvested in the one-fourth, one-half, and three-fourths stages of bloom.

## REVIEW OF LITERATURE

A brief survey has been made of literature pertaining to the stage of maturity, chemical composition, and feeding value and palatability of different cuttings of alfalfa.

### STAGE OF MATURITY

Cottrell (9), Ten Eyck (39), Clarke (7) and Coburn (8) recommend cutting alfalfa in the one-tenth bloom stage for general purpose hay. McCampbell (28), of the Kansas station, found hay from alfalfa cut in full bloom to be most suitable for horses at hard work. On the basis of later work at that station (34) it is recommended to harvest first cutting alfalfa in the one-tenth bloom stage and to delay successive cuttings in the same season until the crop reaches full bloom or nearly so.

Foster and Merrill at the Utah station (11), Harcourt in Canada (16), and Snyder and Hummel (37) at the Minnesota station favor the one-third stage of bloom. Freeman (12) observes that the one-fifth to one-third stage of bloom is best under Arizona conditions, while Voorhees (40) considers that the fourth bloom yields the best quality hay.

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<sup>2</sup> Thanks are due to Prof. H. Hackedorn, head of the Department of Animal Husbandry, for helpful suggestions; to H. F. Singleton, agronomist of the Washington Irrigation Branch Station, for supplying hay samples; and to the Division of Chemistry, in whose laboratory the writer did the analytical work.

<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 381.

Hughes (21) at the Iowa station suggests cutting alfalfa when one-tenth to one-fourth in bloom, while Hunt (22) states that in irrigated regions the practice is to cut when one-tenth to one-third of the total flowers are formed. He adds that the crop should be cut as soon as the lower leaves begin to turn yellow, regardless of the stage of bloom.

Kiesselbach (23) at the Nebraska station notes that too frequent cutting results in weakening of alfalfa plants, and recommends the first-bloom stage for best hay. Bennett (5) also advises cutting when a few blooms appear over the field. At the Ohio station Williams and Kyle (43) observed injury to alfalfa from cutting too early before first bloom or from cutting too late after the crop was too mature. A similar observation is made by Porter and Dynes (32) in North Dakota, and Stewart (38) also points out that too early cutting is accompanied by too much danger to the stand.

Singleton (36) at the Washington station reported for a total of three cuttings average yields of 6.86, 6.71, and 5.83 tons of the three-fourths, one-half, and early-blooming stages, respectively. Cutting off new shoots did not affect the yield or hurt the stand.

Russell and Morrison in Wisconsin (33) recommended the near-full-bloom stage for cutting alfalfa without getting the hay too coarse. Early cutting thinned the stand, weakened the plants, and permitted the encroachment of weeds. Cutting off crown shoots, contrary to common opinion, did not injure the stand. Headden (19) at the Colorado station also favors the full-bloom stage.

According to Piper (31) the general practice in America is to cut alfalfa for hay shortly after the first blossoms appear, except for horses, in which case the full-bloom stage is considered best, as earlier cuttings generally prove too laxative.

At the Michigan station (35) Shoesmith states that alfalfa should be cut when it begins to bloom and new shoots appear at the crown, while J. E. Wing (44) writes that usually when alfalfa is ready to be cut, it will be partly in bloom, sometimes being much more advanced than at other times. It is further suggested that alfalfa should be cut for hay whenever it is ready to make new growth.

The preceding recommendations favor the early bloom stages, 7 being in favor of the one-tenth bloom, 4 favoring the early bloom, 2 the one-fourth bloom, and 4 the one-third bloom stage. The work of the Wisconsin and Colorado stations appears to be at variance in that the full-bloom stage is favored.

#### CHEMICAL ANALYSIS AND DIGESTIBILITY

A survey of the chemical composition of hays of the first, second, and third cuttings of alfalfa was made. Only the averages for 16 determinations chosen from sources reporting three cuttings per year were used. The stage of maturity was not considered in averaging these data, since in many of the investigations just the cutting irrespective of maturity is considered. For comparative purposes all determinations were recalculated to a 93 per cent dry-matter basis, this figure being used because it approximates the moisture content of well-cured hays produced in the Yakima section of the State of Washington. The results of the survey are shown in Table 1.

TABLE 1.—Average percentage composition of first, second, and third cuttings of alfalfa hay <sup>a</sup>

[93 per cent dry-matter basis]

Cutting	Crude protein (N×6.25)	Crude fiber	Nitrogen-free extract	Ether extract	Crude ash
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
First.....	14.37	31.96	35.55	1.94	9.15
Second.....	14.24	33.49	34.64	1.54	8.79
Third.....	14.21	31.15	36.64	1.93	9.07

<sup>a</sup> This table was compiled from analyses obtained from the following references: 1, p. 171; 11, p. 168, 171, 174; 13, p. 8; 17, p. 31-32; 18, p. 6, 9; 20, app. Table II; 24, p. 31-37; 25, p. 7; 26, p. 108; 30, p. 16, 31-32; 41, p. 14-15; 42, p. 58.

Since the average figures do not indicate the variations in composition, the extremes are reported separately. For the first cutting the protein varied from 11.62 to 18.69 per cent; fiber from 26.32 to 42.46 per cent; and the nitrogen-free extract from 27.09 to 39.99 per cent.

The crude protein varied for the second cutting from 11.43 to 17.17 per cent; crude fiber from 26.42 to 41.77 per cent, and nitrogen-free extract from 22.61 to 39.53 per cent. For the third cutting the variation in protein content was from 12.01 to 14.92 per cent; in fiber from 27.29 to 37.47 per cent; and in nitrogen-free extract from 22.61 to 39.53 per cent.

The average figures in Table 1 show very little difference in composition of hays from the three cuttings of alfalfa. A much greater variation is secured in the separate determinations of each cutting.

The average results of a survey of the digestion experiments with hays produced from the three cuttings of alfalfa when these were fed to ruminants are summarized in Table 2.

TABLE 2.—Average coefficients of digestibility of hay from three cuttings of alfalfa <sup>a</sup>

Cutting	Dry matter	Crude protein (N×6.25)	Crude fiber	Nitrogen-free extract	Ether extract	Crude ash
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
First.....	60.45	71.70	43.23	72.46	37.63	49.24
Second.....	61.79	75.90	44.42	73.86	44.32	51.91
Third.....	55.82	68.68	38.35	68.25	39.40	44.13

<sup>a</sup> The coefficients are averages of those reported in the following publications: 15, p. 26; 20, app. Table II; 24, p. 31-37; 25, p. 28-29; 27, p. 16.

The variations in coefficients from the different sources are not great. The averages show second-cutting hay to be quite well digested. Markedly lower digestibility for the third-cutting hay can be noticed. From Tables 1 and 2 the content of digestible nutrients in the hays from three cuttings was computed. The results are shown in Table 3.

TABLE 3.—Average digestible nutrients in first, second, and third cuttings of alfalfa

Cutting	Crude protein (N×6.25)	Crude fiber	Nitrogen-free extract	Ether extract	Total digestible nutrients	Nutritive ratio 1 to—
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	
First.....	10.30	13.82	25.76	0.73	51.52	4.00
Second.....	10.81	14.88	25.59	.82	53.13	3.90
Third.....	9.76	11.95	25.01	.76	48.43	3.96

Table 3 shows that the third-cutting hay contains the least total digestible matter as well as the least digestible crude protein. It forms an excellent basis for later comparisons.

#### FEEDING VALUE AND PALATABILITY

##### RESULTS WITH DAIRY COWS

At the Utah Station (6) the second-crop alfalfa hay proved at least equal to the other crops for milk production. In all cases the cows took more readily to first and third-crop hay than they did to the second-crop hay, and at times were very reluctant about feeding on second-crop hay.

##### RESULTS WITH SHEEP

During the years 1922 to 1926 the lamb-feeding experiments at the Washington Irrigation Branch Experiment Station each year included studies with first, second, and third cuttings of northern grown common alfalfa hay, which were made at the one-half bloom stage of maturity. A daily ration of three-fourths of a pound of grain was fed each lamb in addition to all the hay it would consume. During one of these years wheat was substituted for corn. Descriptions of the lambs, the experimental methods, and the three years' results are reported in a previous publication (14). The data in Table 4, which are based on four years of field work, fail to show any differences over such a period of time in the feeding results with different cuttings of alfalfa. There was no great difference in condition of the lambs fed on different cuttings when they were ready for market.

TABLE 4.—Comparative value of first, second, and third cuttings of alfalfa hay in feeding lambs

[Figures are based upon 60-day experiments for four years]

Items	Cutting of alfalfa hay		
	First	Second	Third
Number of lambs.....	225	175	175
Initial weight (pounds).....	69.11	69.48	70.08
Final weight (pounds).....	85.92	85.84	86.95
Total gain 60 days (pounds).....	16.81	16.36	16.87
Average daily gain (pound).....	.282	.275	.283
Average daily ration:			
Grain fed (pound).....	.75	.75	.75
Hay fed (pounds).....	2.67	2.65	2.69
Hay refused (pound).....	.49	.42	.45
Feed required per cwt. gain:			
Grain (pounds).....	268	275	267
Hay fed (pounds).....	953	972	957
Hay refused (pounds).....	175	154	160
Hay consumed (pounds).....	778	818	797
Percentage of hay refused.....	18.36	15.84	16.72

The lambs had access to good clean hay. It would naturally be assumed that the most palatable hay would be consumed in greatest quantities. However, Table 4 shows a uniform consumption of all three cuttings, so that the four-year study fails to reveal any great differences in palatability. In fact, had all three lots of lambs been fed the same cutting of hay, they might have been expected to show as great differences as were observed between the groups fed different cuttings.

During the first year's trials two very uniform groups of lambs, each containing 50 head, were fed identical rations, with the result that differences in gains greater than those reported in the experiment covered by Table 4 were observed. The single results of each year show that during two years the feeding gains with second-cutting hay were slightly less than the average for all cuttings. The experiments during one year showed no difference between the feeding gains for second-cutting hay and those for other cuttings, whereas in experiments for another year second-cutting hay showed to best advantage. The results of lamb-fattening experiments with alfalfa fail to support the widespread belief that second-cutting hay is enough lower in nutritive value to be discriminated against in the open market.

#### RESULTS WITH BEEF CATTLE

Work at the Utah station (30) shows that when steers were fed alfalfa with or without grain, the most rapid gains were secured on the early-cut hay and the lowest on the late-cut hay, the rating being as follows: Early cut hay 100, medium-cut hay 77, and late-cut hay 68. A similar rating according to value of cutting for beef production showed first cutting as 100, second cutting 75, and third cutting 110. In respect to palatability as measured by pounds of hay actually eaten, first cutting was rated as 100, second 97, and third, 105. Later work at the same station (11) shows that the largest gains by two-year-old steers were made from the early cuttings, and the lowest gains from the late cuttings, the results standing proportionately 100 for early cut hay, 85 for medium-cut hay, and 75 for late-cut hay. The Utah results show the highest feeding value for the third cutting and the lowest for the second, which is in conformity with the earlier work.

Work at Kansas (34) shows that the feeding value of alfalfa hay decreases materially as cutting is delayed, the best hay for feeding beef steers being secured at the bud stage and the poorest at the seed stage.

#### PURPOSE OF THE INVESTIGATION

This investigation was divided into four phases as follows:

(1) A study of the composition of each of the three cuttings of northern-grown common alfalfa at three stages of maturity was made. Alfalfa of the first, second, and third cuttings was harvested in 1923 and 1924, at the one-fourth, one-half, and three-fourths stages of bloom, these stages being determined as far as possible by bud and blossom counts. In addition to a study of the organic nutrients, studies of calcium and phosphorus were made.

(2) Samples of alfalfa hay of the different cuttings and stages were fed to lambs receiving no other feed. Coefficients of digestibility for each of the nutrients in alfalfa hay were determined, and from these data the digestible nutrient content of the hay of each cutting was computed.

(3) Nitrogen balance studies were undertaken with the view of determining at what stage of maturity of the alfalfa plant the protein is best utilized.

(4) An interpretation of the nutrition studies in terms of acre yield was made.

## METHOD OF EXPERIMENTATION

## GENERAL PROCEDURE

The studies of each stage of maturity of the three cuttings were made in triplicate, two wethers and one ewe being used each time. After a 10-day preliminary period in which the experimental ration was fed, the lambs were put into the metabolism crates for 10 days, quantitative collection of feces and urine was made, and an accurate record of feed, water, and refuse hay kept. At the end of the 10-day experimental period the three lambs on test were given a 10-day rest, the other series of two wethers and one ewe being placed in the crates. In this manner the nine samples of hay collected in 1923 and the nine in 1924 were tested during the spring of 1924. The same lambs used in studying the 1923 samples were fed the hay of 1924 and in the same order. This eliminates the factor of individuality in comparisons of digestibility of similar hays produced in 1923 and 1924.

The age of the lambs within limits is not a great factor, inasmuch as normal lambs between the ages of 6 and 14 months digest feed equally well (3). In nutrition experiments it is easier to work with sheep than with cattle, and inasmuch as the results of digestion trials with sheep apply also to cattle in most cases, sheep were used in this test. H. P. Armsby (3) states that Wolff, in 1874, compared the results of about 40 German experiments on cattle and sheep, and Jordan and Hall have made similar comparisons of nine American experiments. On the basis of comparisons of this sort it has been generally considered that digestion coefficients obtained with one species of ruminants may be applied to others without material error. The sheep or goat has been the favorite experimental animal. As regards the better grades of roughages, it has been shown that the difference in digestive power between cattle and sheep is not marked.

## METHOD OF SAMPLING

All of the hay fed was first chopped into one-half and three-fourths inch lengths and thoroughly mixed and sampled (10) for analysis. All the feed for any 10-day experimental period was weighed into paper sacks at one time to equalize moisture changes.

As the trials progressed the refuse hay was carefully collected and sampled. During the study of the 1923 samples some waste hay was collected during each trial, while in subsequent studies with hay of 1924 the lambs ate all the hay without waste.

The metabolism crates were cleaned three times during each 10-day period and the feces of each lamb was collected quantitatively, placed in air-tight receptacles, and the complete sample mixed and sampled for study. The urine was collected daily and kept in stoppered glass flasks over toluol. To prevent loss of ammonia, sulphuric acid instead of toluol was at first added to the samples. However, later study showed this to be unnecessary.

## LAMBS AND THEIR BODY WEIGHTS

Six lambs sired by Hampshire rams and out of Lincoln-Merino ewes were selected from a band of range sheep. The different types were selected with care. Four of the lambs were wethers and two were ewes. The accompanying illustration (fig. 1) gives a fairly accurate idea of type.

These lambs were gradually accustomed to the metabolism crates until they could stand on the half-inch mesh-wire screen with practically no discomfort. The lambs were normal and maintained good appetites throughout the complete series of experiments. During

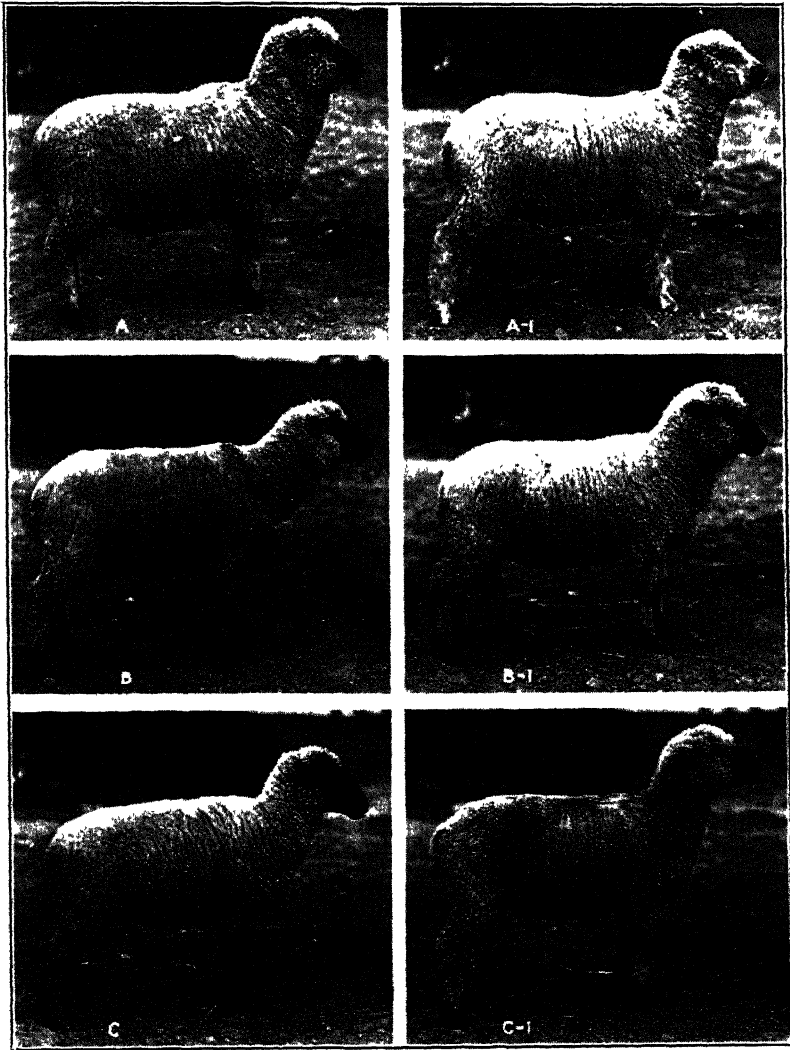


FIG. 1.—The sheep shown in vertical columns are those used in the two series of alfalfa-feeding experiments. The bottom horizontal row shows the ewe lambs, all others being wethers

27 of the 10-day digestion periods the lambs gained weight, whereas they lost weight during 27 other periods. The average loss for the 10-day periods was 2.5 pounds, and the average gain 2.34 pounds per lamb. The average weight of the lambs for the entire period was 82.36 pounds.

## RATIONS FED

The daily ration was fed in three equal portions, the consumption of chopped hay reaching 2.65 pounds (1,200 gm.). This quantity of feed contains 0.20 pounds of digestible crude protein and 1.25 pounds of total digestible nutrients. Such a ration for lambs averaging 82.36 pounds is a medium ration, well above the maintenance requirements. Two grams of salt were added to each feed of hay for which a correction was made. During the preliminary periods free access to salt was given.

The quantity of hay per lamb fed daily does not affect the digestibility (3) when alfalfa alone is fed. However, in mixed rations with grains, the heavier rations are generally less digestible.

The lambs drank 2.37 pounds of water for every pound of hay they consumed. This figure includes evaporation. During the warmer months of July and August, the figure was increased to 3.03 pounds of water.

## ANALYTICAL PROCEDURE

Analyses of feed, feces, and urine were made as the experiment progressed, by methods of analysis adopted by the Association of Official Agricultural Chemists (4). The phosphorus and calcium content of the hay was also determined.

## CHEMICAL ANALYSIS OF FEED SAMPLES

The chemical composition of hay samples which were fed to the lambs during the digestion trials is reported in a succeeding table based on averages for the two years. Another table stressing the cutting rather than the stage of bloom is added. The calcium and phosphorus content of the samples was determined and is given in Table 7.

The composition of the samples of 1923 indicates that there is no marked difference in the hay at one-fourth and one-half bloom stages. At the more mature stage of three-fourths bloom, the hay contains less protein and is somewhat higher in fiber. The first cutting at the one-fourth bloom stage contained only 29.53 per cent fiber in contrast to 40.23 per cent in the third cutting at the three-fourths bloom stage. The third cutting contained only 8.86 per cent of protein. This particular cutting was harvested late in the season and was produced in cooler weather and seems out of line with the rest of the hays.

The analyses of hay samples of 1924 agree in general with those of 1923. There seems to be no clear-cut relationship between fiber content and maturity in the individual samples. At the one-half and three-fourths bloom stages the second and third cuttings contain more fiber. The third cutting at the three-fourths bloom stage again has a low protein content. Averages of all three cuttings at any particular stage show that in 1924 a progressively lower protein content was found in the hays as maturity progressed. The data for ether extract and ash are quite variable.

The data for the two years have been averaged and are included in Table 5. This table brings out in somewhat sharp relief the fact that the protein content of the hays decreases as the plant matures, while the fiber in turn increases.

TABLE 5.—Average composition of first, second, and third cuttings of alfalfa hay when the plant is cut at the one-fourth, one-half, and three-fourths stages of bloom <sup>a</sup>

[Reduced to a 93 per cent dry-matter basis, samples of 1923 and 1924]

Cutting	Crude protein (N×6.25)	Crude fiber	Nitrogen-free extract	Ether extract	Crude ash
One-fourth bloom stage:	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
First.....	11.78	31.96	40.16	2.20	6.90
Second.....	13.40	35.22	34.02	.97	9.40
Third.....	13.96	36.08	33.22	1.04	8.72
Average.....	13.05	34.42	35.50	1.40	8.34
One-half bloom stage:					
First.....	12.07	35.20	37.43	.88	7.43
Second.....	12.98	35.12	36.07	1.27	7.58
Third.....	12.37	36.21	34.98	1.22	8.23
Average.....	12.47	35.51	36.16	1.12	7.75
Three-fourths bloom stage:					
First.....	11.36	34.76	38.60	1.60	6.69
Second.....	11.29	37.77	33.84	1.06	9.04
Third.....	9.06	38.59	36.42	.79	8.16
Average.....	10.57	37.04	36.29	1.15	7.96

<sup>a</sup> Analysis based on stage of bloom at which hay is cut.

A regrouping of the data contained in Table 5 according to cuttings rather than stages of bloom, is reported in Table 6. The two years' data show that ordinarily the first cutting is somewhat lower in protein than the second and third cuttings at the one-fourth and one-half bloom stages. However, as the alfalfa is permitted to mature the differences become less, and the poorest protein hay is secured with the third cutting made at the three-fourths bloom stage.

TABLE 6.—Average composition of first, second, and third cuttings of alfalfa hay when the plant is cut at the one-fourth, one-half, and three-fourths stages of bloom <sup>a</sup>

[Reduced to a 93 per cent dry-matter basis, samples of 1923 and 1924]

Stage of bloom	Crude protein (N×6.25)	Crude fiber	Nitrogen-free extract	Ether extract	Crude ash
First cutting:	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
One-fourth.....	11.78	31.96	40.16	2.20	6.90
One-half.....	12.07	35.20	37.43	.88	7.43
Three-fourths.....	11.36	34.76	38.60	1.60	6.69
Average.....	11.74	33.97	38.73	1.56	7.01
Second cutting:					
One-fourth.....	13.40	35.22	34.02	.97	9.40
One-half.....	12.98	35.12	36.07	1.27	7.58
Three-fourths.....	11.29	37.77	33.84	1.06	9.04
Average.....	12.56	36.04	34.64	1.10	8.67
Third cutting:					
One-fourth.....	13.96	36.08	33.22	1.04	8.72
One-half.....	12.37	36.21	34.98	1.22	8.23
Three-fourths.....	9.06	38.59	36.42	.79	8.16
Average.....	11.80	36.96	34.87	1.02	8.37

<sup>a</sup> Analysis based on cutting of hay.

The averages for the first, second, and third cuttings in the preceding table when compared with the data of Table 1 show that the hays studied contain somewhat less protein and more fiber than would be expected. Somewhat less ether extract and ash was found in these samples. The calcium and phosphorus content of the same hay samples under discussion was determined. The results of these determinations are contained in Table 7.

TABLE 7.—*Per cent calcium and phosphorus contained in alfalfa hay studied*

[7 per cent moisture basis]

Cutting	Calcium			Phosphorus		
	1923	1924	Average	1923	1924	Average
One-fourth bloom:	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
First.....	1.371	1.234	1.302	0.164	0.145	0.154
Second.....	1.331	1.025	1.178	.203	.177	.190
Third.....	1.174	1.416	1.295	.148	.218	.183
Average.....	1.292	1.225	1.258	.172	.180	.176
One-half bloom:						
First.....	1.610	1.347	1.478	.162	.145	.154
Second.....	1.392	1.196	1.294	.190	.169	.180
Third.....	1.354	1.546	1.450	.176	.114	.145
Average.....	1.452	1.363	1.407	.176	.143	.160
Three-fourth bloom:						
First.....	1.449	1.326	1.388	.138	.165	.152
Second.....	1.352	1.189	1.270	.142	.126	.134
Third.....	1.312	1.240	1.276	.093	.106	.100
Average.....	1.371	1.252	1.311	.124	.132	.129

The calcium content of alfalfa is seven to eleven times greater than its phosphorus content. Plants cut at the one-half bloom stage contain more calcium than similar samples of alfalfa cut at the one-fourth and three-fourths stages of bloom. The data fail to show any significant relationship between the number of the cutting and calcium content. The results with samples of 1923 show that first cutting at any of the stages contains more calcium than the second, and the second more than the third. Results for 1924 show that the first cutting contains more calcium than any of the others at the three-fourths stage of bloom only. At all three stages of bloom that year the second cutting contained less calcium than the third.

The results with phosphorus are quite variable and no conclusions seem warranted in respect to the number of the cutting and phosphorus content. The peculiarly low phosphorus content of the third cutting made at the three-fourths stage of bloom is quite striking in view of the fact that it is also low in protein. Much nutritive value is lost by the hay crop when it is leached by rain or dews. At Ohio (2) it was demonstrated that 50 per cent of the nitrogen, 75 per cent of the phosphorus, 90 per cent of the potassium, and 40 per cent of the calcium can be leached out of the hay crop by artificial means.

The calcium and phosphorus content of alfalfa hay is of considerable interest to the feeder. Alfalfa cut at the one-half bloom stage yields most calcium from its three cuttings, while the one-fourth bloom stage is richest in phosphorus. It is recognized that the con-

tent of the mineral elements in any feed is not the only indicator of its value in supplying minerals for body functions. Sunlight and one or more of the vitamins are intimately associated with the utilization of these mineral elements.

#### CHEMICAL ANALYSIS OF FECES

The average analysis of the feces from the first 27 digestion trials when hay samples of 1923 were fed are reported separately from the average of the second series of 27 digestion trials, during which period samples of 1924 were fed. For comparative study the figures were computed to the average moisture content of all feces studied. These figures are shown in Table 8.

TABLE 8.—Average composition of feces when lambs were fed chopped alfalfa hay

Series	Water	Crude protein (N×6.25)	Crude fiber	Nitrogen-free extract	Ether extract	Crude ash
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Percent</i>	<i>Per cent</i>
First.....	58.42	4.45	18.11	12.50	1.11	5.37
Second.....	58.42	4.25	18.72	11.45	1.86	5.30

Each lamb excreted on the average 3.05 pounds of feces per day per 100 pounds live weight, when a medium ration of chopped alfalfa hay was fed. For every pound of dry matter actually consumed, 0.47 pound of fecal dry matter was excreted, which is equal to 1.13 pounds of feces, with an average moisture content of 58.42 per cent.

The digestion trials show that an average of 44.79 per cent of the consumed organic matter in the form of alfalfa hay is excreted in the feces. Minimum and maximum figures of 28.91 and 59.88 per cent were noted.

#### COEFFICIENTS OF DIGESTIBILITY

When the results of ordinary digestion experiments are corrected for nitrogenous and other excretory products found in the feces, an approximation of true digestibility is obtained. Armsby (3) states that the intestinal products found in the feces are, in effect, part of the cost of digesting the feed. He states that they represent the "wear and tear" of the digestive organs. The difference, then, between feed and feces will show the net gain to the animal from the digestion of the feed. From this point of view one may speak of the digestibility as ordinarily determined as the *apparent digestibility*. The subsequent studies are based upon coefficients of apparent digestibility.

The results of a single digestion trial are reported in detail in Table 9. Fifty-four such experiments form the basis of the digestibility studies, but lack of space precludes their tabulation in full. The tables of average coefficients of apparent digestibility bring out in full detail the results of these experiments.

TABLE 9.—*Results of trial 1 secured with wether lamb No. 339. The first cutting of 1923 in the one-fourth bloom stage was fed for 10 days*

	Dry matter	Crude protein	Crude fiber (N×6.25)	Nitrogen-free extract	Ether extract	Organic matter
Alfalfa hay fed (grams).....	10,646	1,427	3,380	4,675	418	9,900
Alfalfa hay refused (grams).....	799	76	341	326	16	749
Total hay consumed (grams).....	9,847	1,351	3,039	4,349	402	9,151
Feces (grams).....	3,717	393	1,592	1,066	74	3,125
Total digested (grams).....	6,130	958	1,447	3,283	328	6,026
Coefficients of digestibility.....	62	71	48	76	82	66

Since all the feed for any 10-day digestion trial was weighed into paper sacks at one time, a very accurate determination of the dry-matter intake was possible. The feed rack shown in Figure 2 was so constructed that all feed scattered by the lambs could be recovered. The urine was also collected quantitatively, and its nitrogen content serves as the basis of subsequent nitrogen-metabolism studies.

The coefficients of digestibility represent, by a single number, results of many complex physical and chemical changes. In a subsequent table these coefficients are reported as whole numbers, since even the whole numbers are at best only relative.

The negative coefficients for ether extract signify that the fecal ether extract exceeded that contained in the feed consumed. Only anhydrous ether was used in the determinations. Many of the bile salts are soluble in ether and for that reason even the positive coefficients of digestibility are not reliable. Inasmuch as considerable ash is excreted in the large intestine, fecal ash is made up of indigestible ash as well as excretory ash. For that reason digestion studies with ash are meaningless and are not reported.

The dry matter of first-cutting hay is digested better than the dry matter of either of the other two cuttings, at all three stages of maturity. The protein of the third-cutting hay is not digested as well as that of the first and second cuttings. At the three-fourths bloom stage the digestibility of protein is 30 per cent less than for the other two cuttings, and a considerably lower digestibility of the dry matter and fiber is noted.

The results for 1924 show a lower digestibility for dry matter and crude fiber than do those of 1923. Lambs consumed the 1924 hay completely, while some stems remained in the feed troughs during each trial when the hays of 1923 were fed. The intake of fiber per lamb therefore was greater in the first case and helps explain the somewhat lower digestion coefficients. The coefficients for the third cutting feeding experiments at the three-fourths bloom stage are not as much out of line as those secured for the identical stage produced in 1923.

The results for the two years were averaged and are summarized in Table 10.

The table shows that at the one-fourth and one-half bloom stages there is no marked difference in the digestibility of dry matter. This is true also of the first and second cuttings made at the three-fourths bloom stage. However, the third cutting at the three-fourths bloom stage appears to have a much lower digestibility. A coefficient of 46, which is the average of six determinations, appears to be 9 and 6 points lower than the coefficients for first and second cuttings, respectively.

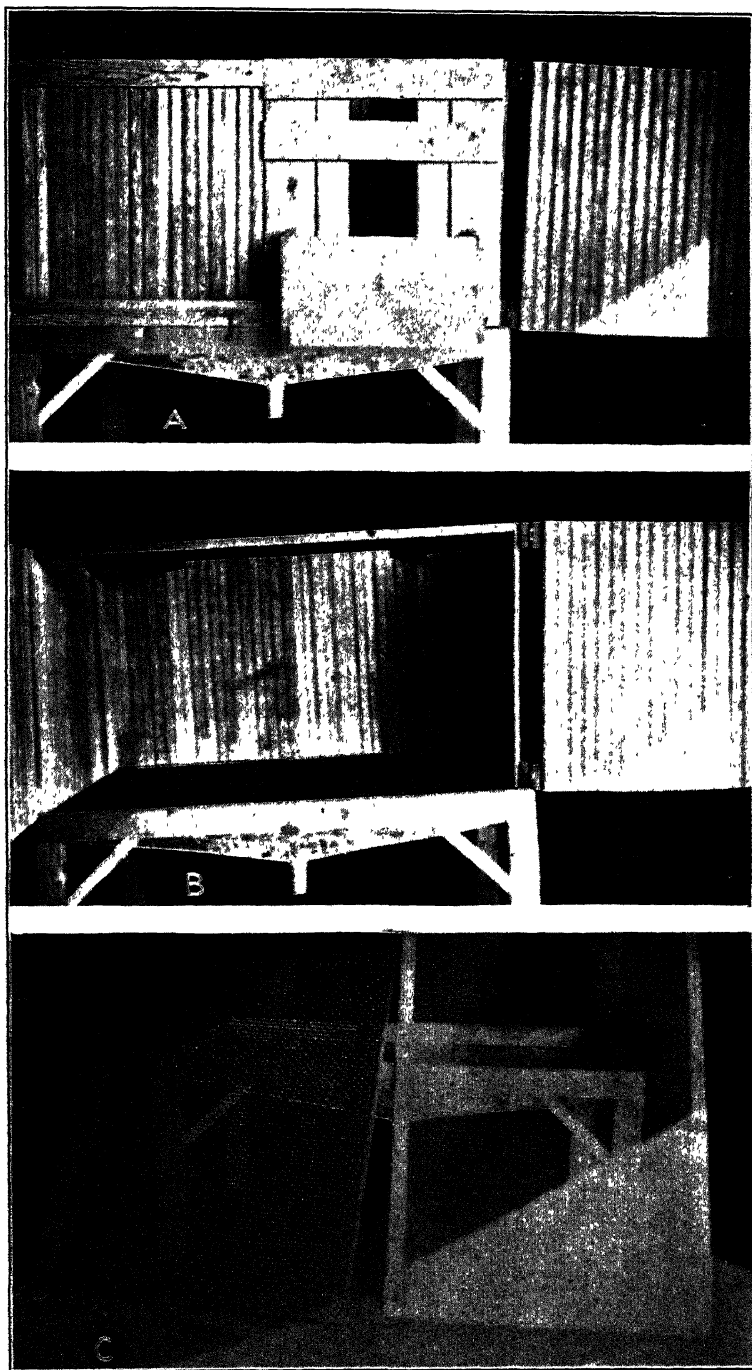


FIG. 2.—A, Metabolism crate with the feed rack in place. B, An open view of the same crate. C, Type of screens used in the construction of these crates

TABLE 10.—Average coefficients of apparent digestibility secured with irrigated alfalfa hay

[Produced in 1923 and 1924 <sup>a</sup>]

Cutting	Dry matter	Crude protein (N×6.25)	Crude fiber	Nitrogen-free extract	Ether extract	Organic matter
One-fourth bloom stage:						
First.....	54	62	40	68	28	57
Second.....	55	68	48	63	—39	56
Third.....	54	67	46	64	4	56
Average.....	54	66	45	65	-----	56
One-half bloom stage:						
First.....	58	66	52	68	—38	60
Second.....	54	69	44	64	14	56
Third.....	53	62	47	67	-----	54
Average.....	55	66	48	66	-----	57
Three-quarter bloom stage:						
First.....	55	62	45	67	38	58
Second.....	52	60	44	62	—6	53
Third.....	46	45	38	64	-----	46
Average.....	51	56	42	64	-----	52

<sup>a</sup> Each stage of maturity was studied in triplicate, so that each coefficient in the table is the average of six determinations.

The digestibility studies with crude protein favor the one-fourth and one-half bloom stages. These appear to produce hay of a protein content that is equally well digested. However, at the three-fourths bloom stage protein of first and second cuttings is similarly digested, the coefficients for these cuttings being 62 and 60, respectively. The third cutting at this stage has a coefficient of only 45. It is true that this third cutting, made so late in the season, is subject to cooler temperature, more humidity during its growth, and a greater leaf loss during its curing period. A tougher fiber seems to develop also, which appears to exert a secondary influence in depressing the digestibility of protein and other nutrients.

The fiber at the one-half bloom stage is better digested than at the one-fourth bloom stage, although the difference is not great. At the three-fourths bloom stage, particularly in the third cutting, the fiber seems to be tougher in nature and lower coefficients are noted. The third cutting which gives a coefficient of 38, ranks last, the first and second cuttings at the three-fourths bloom stage having coefficients of 45 and 44.

The nitrogen-free extract is apparently equally well digested at all three stages of bloom.

#### DIGESTIBLE NUTRIENTS OF ALFALFA HAY

The difference in digestible nutrients in the hays of 1923 and 1924 can be explained partly on the basis that some stems were not consumed by the lambs fed the hays produced in 1923, while in subsequent trials the lambs consumed their feed completely. In such cases the waste stems were analyzed and their nutrient content subtracted from the nutrients in the hay fed. The hay actually consumed was more digestible per unit weight than if no stems had

been wasted; however, the waste was small and fairly uniform for each trial, and the relative digestibility of the different samples should not be greatly affected.

A comparison of the two years' results shows a lower digestibility of dry matter in 1924. The third cutting of 1923 made at the three-fourths stage of bloom is shown to possess low nutritive value, being especially low in digestible crude protein and total digestible nutrients. A similar cutting produced in 1924, although rather low in digestible protein, contained as much digestible nutrients as any of the cuttings produced that year.

Averages for all three cuttings produced in 1923 show that hays of almost equal nutritive value are produced at the one-fourth and one-half bloom stages, but that delaying cutting until the three-fourths stage of bloom lowers the nutritive value.

The 1924 average results show that the one-fourth bloom stage of maturity is to be preferred when hays with a high protein content are desired. Progressively less digestible protein is found in the hays produced from plants in the one-half and three-fourths stages of bloom. However, the greatest quantity of total digestible nutrients is secured when the hay is cut at the one-half bloom stage.

The average results (Table 11) based on two years' work show that a high content of digestible crude protein is secured at the one-fourth bloom stage, and a rather lower protein content by permitting the plants to mature. At the one-half bloom stage the plants produce a hay that contains the maximum of digestible nutrients.

TABLE 11.—*Digestible nutrients per 100 pounds when alfalfa is cut at the one-fourth, one-half, and three-fourths bloom stage*

[Average of 1923 and 1924]

Cutting	Dry matter	Crude protein (N×6.25)	Crude fiber	Nitrogen-free extract	Ether extract	Total digestible nutrients
One-fourth bloom stage:						
First.....	49.76	7.35	12.56	27.37	1.49	50.63
Second.....	51.15	9.04	16.92	21.39		47.35
Third.....	50.68	9.50	16.48	21.15	.19	47.57
Average.....	50.53	8.63	15.32	23.30		48.52
One-half bloom stage:						
First.....	53.94	7.98	18.32	25.22		51.52
Second.....	49.76	8.94	15.48	23.14	.18	47.96
Third.....	49.29	7.74	17.10	23.38	.12	48.51
Average.....	51.00	8.22	16.97	23.91		49.33
Three-fourths bloom stage:						
First.....	51.15	6.98	15.73	25.85	.66	50.03
Second.....	47.90	6.82	16.40	21.15	.01	44.40
Third.....	42.32	4.06	14.43	23.56		42.07
Average.....	47.12	5.96	15.52	23.52		45.50

Table 12 is a rearrangement of the data reported in Table 11 and aims to bring out differences between first, second, and third cuttings, rather than to emphasize the stages of maturity. The table shows that although first-cutting hay ranks only second in digestible crude protein, it excels the others in digestible nutrients. Second-cutting hay contains the highest average content of digestible crude protein. The average data when compared with that in Table 3 show a lower content of digestible crude protein and total digestible nutrients.

TABLE 12.—*Digestible nutrients per 100 pounds in first, second, and third cuttings of alfalfa hay when cut at the one-fourth, one-half and three-fourths stages of bloom*

[Averages of 1923 and 1924 experiments]

Stage of bloom	Dry matter	Crude protein (N×6.25)	Crude fiber	Nitrogen-free extract	Ether extract	Total digestible nutrients
First cutting:						
One-fourth.....	49.76	7.35	12.56	27.37	1.49	50.63
One-half.....	53.94	7.98	18.32	25.22	-----	51.52
Three-fourths.....	51.15	6.98	15.73	25.85	.66	50.03
Average.....	51.62	7.44	15.54	26.15	-----	50.73
Second cutting:						
One-fourth.....	51.15	9.04	16.92	21.39	-----	47.35
One-half.....	49.76	8.94	15.48	23.14	.18	47.96
Three-fourths.....	47.90	6.82	16.40	21.15	.01	44.40
Average.....	49.60	8.27	16.27	21.89	-----	46.57
Third cutting:						
One-fourth.....	50.68	9.50	16.48	21.15	.19	47.57
One-half.....	49.29	7.74	17.10	23.38	.12	48.51
Three-fourths.....	42.32	4.08	14.43	23.56	-----	42.07
Average.....	47.33	7.11	16.00	22.70	-----	46.05

The average figures for the third cutting are considerably lower because of the third cutting made at the three-fourths stage of bloom, which appears to be out of line with the rest of the hays.

The digestible nutrients per ton reported in Table 13 are of interest to the stockman who must buy hay in the open market. The table shows that first-cutting alfalfa contains the largest quantity of digestible nutrients per ton, while the second cutting is richer in digestible protein. The average results for the third cutting are considerably lowered, due to the inclusions of the late third cutting in the three-fourths stage of bloom. With this stage omitted from the averages, third-cutting hay compares favorably with second-cutting hay.

When the average of digestible nutrients at all the stages of first cutting are considered as 100 per cent, second cutting rates 92, and third cutting 91 per cent. (See Table 13.)

TABLE 13.—*The nutritive value of a ton of alfalfa hay as affected by the stage of maturity of the plant and the number of the cutting*

[Digestible nutrients in pounds per ton, basis of two years' results]

Stage of bloom	Dry matter	Crude protein (N×6.25)	Crude fiber	Total digestible nutrients
First cutting:				
One-fourth.....	995	147	251	1,013
One-half.....	1,079	160	366	1,030
Three-fourths.....	1,023	139	315	1,001
Average.....	1,032	149	311	1,015
Second cutting:				
One-fourth.....	1,023	181	338	947
One-half.....	995	179	320	959
Three-fourths.....	958	136	325	888
Average.....	992	165	329	931
Third cutting:				
One-fourth.....	1,014	190	330	951
One-half.....	986	155	342	970
Three-fourths.....	846	82	289	841
Average.....	949	142	320	921

## YIELD OF DIGESTIBLE NUTRIENTS PER ACRE

At the Washington Irrigation Branch Station (36) yield records kept over a period of six years show that when alfalfa is cut in the three-fourths bloom stage a maximum tonnage of air-dried hay to the acre is secured. However, the one-half bloom stage is a close second, and is given preference, since cutting alfalfa at the three-fourths stage of bloom prolongs the growing season so that the third cutting is produced under less favorable growing conditions. As a result the yield of this last cutting is lowered considerably. Cutting at the one-fourth bloom stage seems impractical in view of the lower yields. The six-year average shows the yield per acre for all three cuttings to be 5.83, 6.71, and 6.86 tons at the one-fourth, one-half, and three-fourths stages of bloom, respectively.

On the basis of these yield records and the digestion experiments, the digestible nutrients per acre have been computed and are shown in Table 14.

TABLE 14.—*Digestible nutrients produced per acre of alfalfa by three cuttings at the one-fourth, one-half, and three-fourths stages of bloom*

[Based upon digestion trials with two years' study of hay samples, and six-year yield data]

Cutting	Air-dried hay <sup>a</sup>	Digestible nutrients per acre					
		Dry matter	Crude protein (N×6.25)	Crude fiber	Nitrogen-free extract	Ether extract	Total digestible nutrients
One-fourth bloom stage:	Pounds	Pounds	Pounds	Pounds	Pounds	Pounds	Pounds
First.....	4,180	2,080	307	525	1,144	62	2,116
Second.....	4,000	2,046	362	677	856	—	1,894
Third.....	3,480	1,764	331	574	736	7	1,655
Total.....	11,660	5,890	1,000	1,776	2,736	—	5,665
One-half bloom stage:							
First.....	5,320	2,870	425	975	1,342	—	2,741
Second.....	4,460	2,219	399	690	1,032	8	2,139
Third.....	3,640	1,794	282	622	851	4	1,766
Total.....	13,420	6,883	1,106	2,287	3,225	—	6,646
Three-quarter bloom stage:							
First.....	5,800	2,967	405	912	1,499	38	2,902
Second.....	5,060	2,424	345	830	1,070	1	2,247
Third.....	2,860	1,210	117	413	674	—	1,203
Total.....	13,720	6,601	867	2,155	3,243	—	6,352

<sup>a</sup> Washington Bulletin 209 (36, p. 14, Table 4), recomputed in pounds.

The digestible crude protein per acre of alfalfa is quite variable and is influenced by the number of the cutting and the stage of maturity of the plant. Only 117 pounds of digestible crude protein was produced to the acre in the third cutting of alfalfa harvested at the three-fourths stage of bloom, while a maximum of 425 pounds was secured with the first cutting in the one-half bloom stage. The first cutting at the three-fourths bloom stage produced 405 pounds.

The yield of hay in pounds to the acre appears to have no relationship to its content of digestible crude protein, and in several instances the lower yields of hay contained a larger quantity of this valuable nutrient. The maximum total protein production to the acre, based on all three cuttings, is secured at the one-half bloom stage of matur-

ity, the yield being 1,106 pounds. At the one-fourth bloom stage of maturity all three cuttings produced 1,000 pounds. The difference is not great, but the one-half bloom stage is to be preferred because it also yields a greater quantity of total digestible nutrients.

The production of digestible nutrients to the acre is of especial interest, since recent studies with dairy cows (29) indicate that existing figures of total digestible nutrients of feeds are a very good measure of the relative value of such feeds as sources of nutritive energy under practical conditions.

The highest seasonal yield of total digestible nutrients was secured at the one-half bloom stage, the three-fourths bloom stage ranking second. From the standpoint of total yield of hay, yield of digestible nutrients, and yield of digestible crude protein, it seems impractical to cut alfalfa at the one-fourth stage of bloom under the conditions of irrigation farming in the Yakima Valley.

The first cutting of alfalfa at the three-fourths bloom stage yielded the maximum of total digestible nutrients. However, the third cutting at this stage yielded less than half as much as the first. Second-cutting hay produces more total digestible nutrients and digestible crude protein than the third cutting at all stages.

Table 14 shows that the one-half bloom stage of maturity of the alfalfa plant is to be preferred to the earlier and later stages in respect to the yield of the several digestible nutrients.

#### YIELD OF CALCIUM AND PHOSPHORUS PER ACRE AND PER TON OF HAY

The calcium and phosphorus content in the three cuttings of alfalfa cut for hay in different stages of maturity is not only of general interest to the livestock man, but also to the hay grower concerned in knowing how much of these elements are removed from his soil when the hay crop is sold to be fed elsewhere.

TABLE 15.—Yield of calcium and phosphorus per acre and per ton of hay by three cuttings of alfalfa cut at the one-fourth, one-half, and three-fourths stages of bloom

Cutting	Air-dried hay per acre	Yield per acre of—		Yield per ton of—	
		Calcium	Phos- phorus	Calcium	Phos- phorus
One-fourth bloom stage:	<i>Pounds</i>	<i>Pounds</i>	<i>Pounds</i>	<i>Pounds</i>	<i>Pounds</i>
First.....	4,180	54.42	6.44	26.04	3.08
Second.....	4,000	47.12	7.60	23.56	3.80
Third.....	3,480	45.07	6.37	25.90	3.66
Total.....	11,660	146.61	20.41		
Average.....				25.17	3.51
One-half bloom stage:					
First.....	5,320	78.63	8.19	29.56	3.08
Second.....	4,460	57.71	8.03	25.88	3.60
Third.....	3,640	52.78	5.28	29.00	2.90
Total.....	13,420	189.12	21.50		
Average.....				28.15	3.19
Three-fourths bloom stage:					
First.....	5,800	80.50	8.82	27.76	3.04
Second.....	5,060	64.26	6.78	25.40	2.68
Third.....	2,860	36.49	2.86	25.52	2.00
Total.....	13,720	181.25	18.46		
Average.....				26.23	2.57

Table 15 shows that at the one-fourth bloom stage all three cuttings contained 146.61 pounds of calcium. Yields of 189.12 and 181.25 pounds at the one-half and three-fourths bloom stages were secured. From the standpoint of total calcium yield per acre the one-half bloom stage is recommended. This is true for phosphorus also. The third cutting made at the three-fourths stage of bloom is quite deficient in calcium and phosphorus in comparison with the other hays. The one-half bloom stage of maturity also shows up to best advantage when the calcium and phosphorus content per ton of hay is computed, as seen in Table 15.

The conclusion that the hay highest in calcium is the best for live-stock feeding is not warranted. It is the hay from which most calcium is assimilated that is of most value in bone development and milk production; and maximum calcium assimilation seems to depend upon the presence of the antirachitic vitamin contained either in the hay itself or in feeds fed with the alfalfa hay. Furthermore, there seems to be a relationship between the care taken in curing the hay and the antirachitic factor. The utilization of calcium from the different hays was not studied. Calcium and phosphorus balance studies would be a valuable addition. However, in a properly constituted ration the alfalfa hay higher in calcium is to be preferred.

#### NITROGEN METABOLISM STUDIES

In Table 16 the column headed "Nitrogen stored per 100 pounds live weight" is used for comparative purposes rather than the nitrogen balance column, since in the former the weight factor is eliminated. The column "Per cent of intake stored" is of value only when the intake of nitrogen per unit of live weight is constant for all lambs. Lambs utilize protein for tissue development in growth and for cellular expansion in fattening, and some protein is used for repair of protein tissue. When all these demands are supplied the excess nitrogen is excreted. For that reason the greater the intake of protein above this requirement, the lower is the percentage utilization of the protein.

Averages of nitrogen-balance studies with alfalfa-hay samples grown in 1923 and 1924 are reported in Table 16. The single year's data are not reported. Three determinations were made each year and the results averaged, so that each figure in the table represents the average of six determinations, except when otherwise specified.

The average of two years' data shows that if all three cuttings are made at the one-fourth bloom stage the largest utilization of nitrogen is secured. The half-bloom stage ranks second while the three-fourths bloom stage is last. The same is true in respect to the nitrogen stored per 100 pounds live weight, which affords a truer basis of comparison in cases in which quite striking differences may be noted. In this respect the results show that the nitrogen utilization of the hay from less mature plants is markedly higher than that from the more mature plants. It decreases progressively as the plants mature.

The purpose of the preceding discussion was to show in what manner the stage of maturity of the alfalfa plant affected the protein utilization when hays of varying degrees of maturity were fed to lambs. A rearrangement of the data with a view to comparing first, second, and third cuttings was made, and Table 17 contains these results.

TABLE 16.—Average results of nitrogen metabolism studies with alfalfa hay fed to lambs at various stages of maturity

[Hay was produced in 1923 and 1924. Totals are for average 10-day period]

Cutting	Initial weight of lambs	Intake of nitrogen in feed per head	Outgo of nitrogen in feces per head	Outgo of nitrogen in urine per head	Balance of nitrogen	Nitrogen stored per 100 pounds live weight	Intake of nitrogen stored
One-fourth bloom stage:	<i>Pounds</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Per cent</i>
First.....	71.68	202.29	76.58	75.44	50.27	71.95	24.72
Second.....	84.00	241.49	78.77	113.28	49.41	58.97	20.41
Third.....	85.42	242.25	75.93	98.86	<sup>a</sup> 73.22	<sup>a</sup> 85.60	28.68
Average.....	80.37	228.67	77.09	95.86	57.63	72.17	24.60
One-half bloom stage.							
First.....	78.04	215.71	73.50	104.73	37.47	47.29	17.20
Second.....	82.54	239.69	74.98	89.08	75.63	93.06	31.77
Third.....	86.17	218.90	82.69	90.07	46.17	53.85	19.29
Average.....	82.25	224.77	77.06	94.63	53.09	64.73	22.75
Three-fourths bloom stage:							
First.....	78.67	202.24	77.29	79.62	45.34	57.26	22.32
Second.....	86.67	199.06	78.88	80.93	39.24	46.13	19.55
Third.....	88.08	157.74	87.02	43.71	<sup>a</sup> 33.57	<sup>a</sup> 39.07	<sup>a</sup> 21.77
Average.....	84.47	186.35	81.06	68.08	39.38	47.48	21.22

<sup>a</sup> Average of 5 determinations. All other figures are averages of 6 determinations, 3 with hays of 1923 and 3 with hays of 1924.

TABLE 17.—Average results of nitrogen metabolism studies with alfalfa hay prepared from plants at the one-fourth, one-half, and three-fourths stages of bloom, arranged according to cuttings

[Hays produced in 1923 and 1924. Total for average 10-day period]

Stage of bloom	Initial weight of lambs	Feed in take of nitrogen in feed per head	Outgo of nitrogen in feces per head	Outgo of nitrogen in urine per head	Balance of nitrogen	Nitrogen stored per 100 pounds live weight	Intake of nitrogen stored
First cutting:	<i>Pounds</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Per cent</i>
One-fourth.....	71.68	202.29	76.58	75.45	50.27	71.94	24.72
One-half.....	78.04	215.71	73.50	104.63	37.46	47.30	17.20
Three-fourths.....	78.67	202.24	77.29	79.62	45.34	57.26	22.31
Average.....	76.13	206.74	75.79	86.59	44.36	58.83	21.41
Second cutting:							
One-fourth.....	84.00	241.48	78.77	113.28	49.40	58.97	20.41
One-half.....	82.54	239.69	74.98	89.08	75.13	93.06	31.77
Three-fourths.....	86.67	199.06	78.88	80.93	39.24	46.13	19.55
Average.....	84.40	226.74	77.54	94.43	54.76	66.05	23.91
Third cutting:							
One-fourth.....	85.42	242.25	75.93	98.86	73.22	85.60	28.69
One-half.....	86.17	218.90	82.70	90.07	46.17	53.85	19.29
Three-fourths.....	88.08	157.75	87.01	43.70	33.58	39.06	21.77
Average.....	86.56	206.30	81.88	77.54	50.98	59.50	23.24

The average data for all first cuttings, made at the one-fourth, one-half, and three-fourths stages of bloom during two years shows a storage of 58.83 gm. of nitrogen each 10-day period per lamb and per 100 pounds live weight. The protein of second-cutting hay is even better utilized, 66.05 gm. of nitrogen being stored. Third-

cutting hay ranks intermediate with a storage of 59.50 gm. The same order pertains in respect to the "Balance of nitrogen" and "Intake of nitrogen stored."

#### GENERAL SUMMARY AND CONCLUSIONS

The main purpose of this investigation was the determination of the chemical composition, digestibility, and protein utilization of first, second, and third cuttings of alfalfa. Each of these three cuttings was studied at the one-fourth, one-half, and three-fourths stages of maturity, and samples grown in 1923 and 1924 were fed to lambs averaging 80 pounds in weight.

Fifty-four digestion and nitrogen-metabolism experiments were carried on and from these studies the digestible nutrients in the various hays were computed, and the utilization of protein determined.

The protein content of alfalfa hay decreased and the fiber content was found to increase, as the plants matured. The third cutting at the three-fourths stage of bloom contained the least protein. Second cutting (average of all stages) contained the highest percentage of protein, followed by the third. First cutting ranked last.

The calcium content of alfalfa was found to be seven to eleven times greater than its phosphorus content. Plants cut at the one-half bloom stage contained more calcium than similar samples harvested at the one-fourth and three-fourths bloom stages. No relationship between maturity and phosphorus content was observed, nor was there any relationship between the number of the cutting and the phosphorus and calcium content of the hays.

The highest content of digestible crude protein and total digestible nutrients was secured from the three cuttings of hay harvested at the one-half bloom stage.

First-cutting alfalfa hay contained the largest percentage of digestible nutrients per ton followed by the second and third cuttings, respectively. Second-cutting hay contained the maximum of digestible crude protein per ton and third cutting the least.

The highest seasonal yield of total digestible nutrients and digestible crude protein to the acre was secured at the one-half bloom stage. In respect to protein, hay at the one-fourth bloom stage ranked second, while the three-fourths bloom stage ranked second in respect to total digestible nutrients.

With increasing maturity of the alfalfa plants a lower utilization of the crude protein was observed.

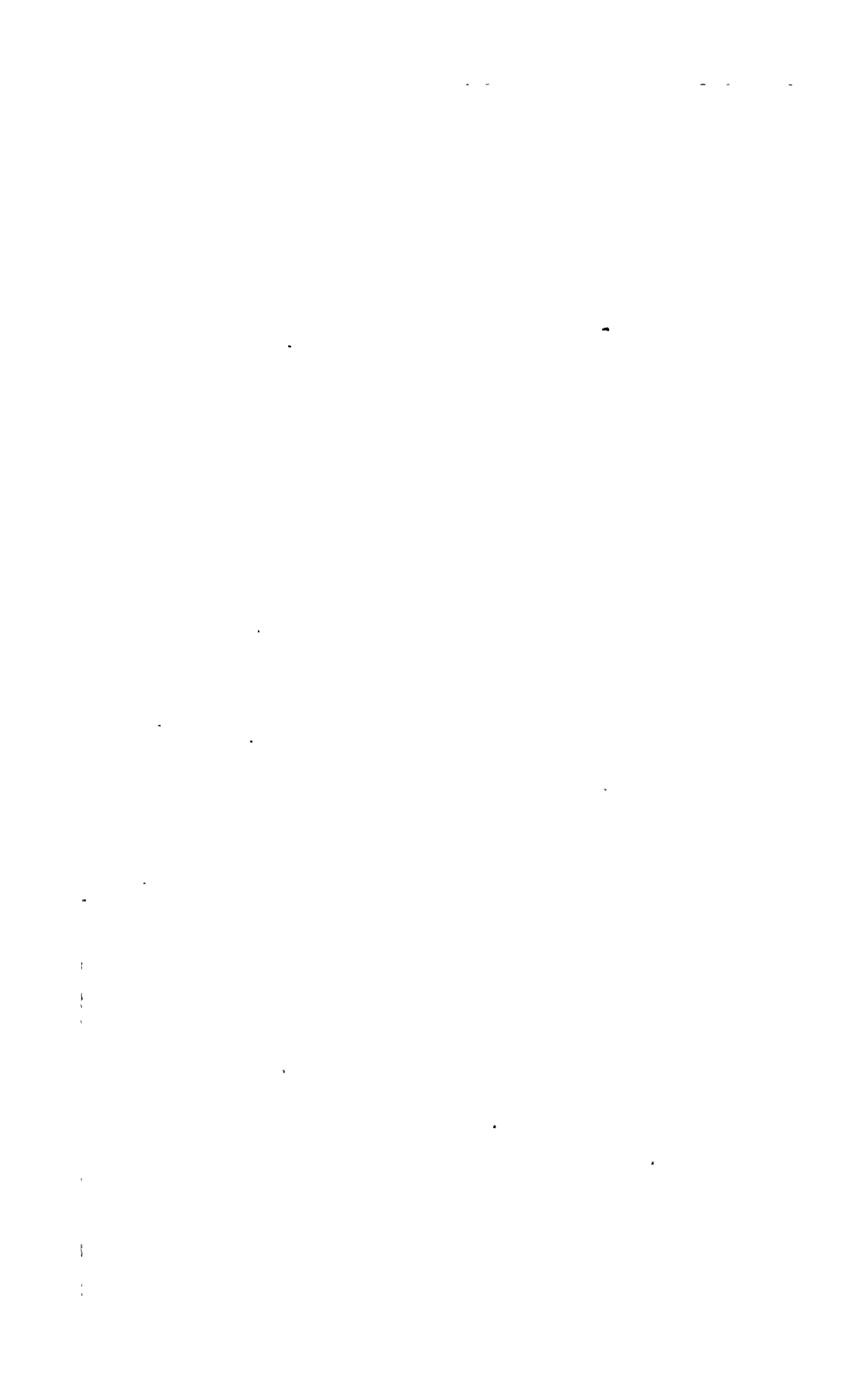
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## THE UTILIZATION OF CARBOHYDRATES BY HONEYBEES<sup>1</sup>

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### INTRODUCTION

In the study of the problem of wintering honeybees it was found that the character and composition of the food plays an important part in successful wintering. It was also suggested from certain findings in that study (68)<sup>2</sup> that not all carbohydrates included in the normal food of bees are utilized and that undigested carbohydrates contribute considerably to the accumulation of feces during the winter confinement to the hive. The presence of an undue amount of feces was found to cause greatly increased muscular activity in bees in winter confinement, since bees do not normally void their feces in the hive, but, if possible, retain them until an opportunity for flight occurs. This increased muscular activity results in the rapid death of individual bees and of the colony as a whole. An especially destructive result is the loss of vitality in those bees which still live. Any condition which causes an accumulation of feces is, therefore, highly undesirable, bringing about the condition known to beekeepers as dysentery.

The apparent difference in the availability of various carbohydrates as food suggested a study of this problem with chemically pure materials, and the work was carried further to include some carbohydrates which bees would not encounter in nature. Since so little is known of the utilization of certain rare carbohydrates, it has seemed worth while to include these to test their availability for an animal which can be controlled easily and for which small quantities of the rare and often expensive carbohydrates would be sufficient to obtain a definite result. In the case of the simple sugars, the question is whether they are capable of absorption and utilization; while for the more complex compounds the question is primarily whether the bees can supply the enzymes necessary for the first breaking down of the compound sugars into simple sugars which must occur before absorption.

It also seems desirable to investigate this subject because of certain peculiarities encountered in practical beekeeping. The darker honeys (except buckwheat honey, in which the dark color is presumably caused by tannin bodies) and honeydews, if given as food during the period of winter confinement, are the materials which cause dysentery.

<sup>1</sup> Received for publication May 17, 1927; issued October, 1927. The feeding experiments as well as some of the calculations herein recorded were made while the author was connected with the Bureau of Entomology. The work was completed and prepared for publication at the New York State College of Agriculture.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 427.

Feces may accumulate to the point where the bees are no longer able to retain them, resulting in spotting of the interior of the hive. The ease with which this condition is relieved if a flight permits the voiding of feces indicates that the presence (pressure?) of feces alone causes the abnormal behavior. The dark honeys and honeydews differ from superior honeys chiefly in their higher dextrin content, suggesting, but by no means proving, that the adult bees are unable to utilize dextrin as food. Earlier investigators have reported the presence of amylase in adult bees, as well as in honeys, and if this enzyme were actually present in the lumen of the alimentary tract, the enzyme capable of hydrolyzing dextrans (dextrases, assuming that there are several enzymes involved in the complete hydrolysis of starch), might also be expected to occur in the lumen of the alimentary tract. If this were the case, a higher percentage of dextrin could hardly be the cause of the great accumulation of unutilized material in the form of feces. The bearing of earlier work on the present problem will be discussed when the experiments on feeding starches and dextrans are detailed.

A short, nontechnical discussion of some of the results of the work herein recorded was published (62) by the author in 1924, to present to beekeepers some of the practical bearings of this problem. Berthoff in a related investigation (10) finds that honeybee larvae are able to utilize dextrans, lactose and galactose, which appears to be untrue for adult bees. The experiments herein recorded were undertaken in connection with the work done by Demuth<sup>3</sup> and the author on the behavior of bees in winter.

#### CARBOHYDRATES AND ENZYME SOURCES INVOLVED IN BEE DIGESTION

Before entering upon a discussion of the experimental work, it seems desirable to suggest complicating factors which may be involved in a study of the utilization of carbohydrate by the honeybee. This is done in order to show the reason for the simple technic here used and to suggest sources of errors which may have entered into the results obtained by others.

The usual method of determining the enzymes present in various organs is to make extracts of the tissues or of digestive juices in water, glycerin, or weak alcohol and to test the action of these extracts on known substances. This method has been used in investigations of the digestive processes of the honeybee. That these methods are open to some criticism will appear in later discussions, especially under the discussion of the utilization of starch by the bees. In making such extracts of the alimentary canal of the honeybee, it appears to be impossible completely to remove the included pollen, which is a prolific source of enzymes. It is also impossible to obtain only the digestive juices or the tissues producing them free of muscle and other tissues, which may introduce an error. It is the belief of the author that such sources of errors are responsible for some of the apparently incorrect results which have been obtained by other investigators.

It is possible to investigate the utilization of various carbohydrates by the honeybee by the methods used in the present instance,

<sup>3</sup> The author wishes to acknowledge the assistance given by George S. Demuth in the first three series of feedings, and by H. S. Paize, in charge of the carbohydrate laboratory, Bureau of Chemistry, for valuable help in planning some of the later series of feedings.

because of the ability of honeybees to live for considerable periods when taking no food other than chemically pure carbohydrates. During the larval period bees consume large quantities of protein, including pollen (56) which are rich in protein, during the latter part of larval life. During adult life worker bees consume large quantities of pollen<sup>4</sup> when engaged in the elaboration of larval food and the feeding of the queens. Otherwise during adult life the workers appear to ingest little or no pollen; and it is known that during winter when they are confined to the hive and when no brood is being reared, bees apparently need little or no pollen and can be carried through the winter (perhaps five months) successfully with no pollen in the hive.<sup>5</sup> Experiments such as are here described would be impossible with any species which requires a mixed diet at all times. To what extent worker bees under such circumstances are drawing on their protein reserve need not concern us at this time.

#### MICROORGANISMS IN THE ALIMENTARY CANAL

The problem of testing the utilization of various carbohydrates may not be one involving simply the production of enzymes by the bees themselves. A considerable number of species of microorganisms occur in the intestine, and attention must be given to such members of this flora as might modify the results. This is important in view of the belief sometimes expressed that certain carbohydrates are hydrolized by the action of enzymes produced not by the mammalian alimentary canal but by microorganisms occurring in it. An even more striking case occurs in termites, in which cellulose is hydrolized by intestinal protozoa. There is, of course, the possibility that some of the bacterial species in the bee intestine produce enzymes which are involved in the hydrolizing action in bee digestion.

In considering this question one is concerned chiefly with those microorganisms which are regularly found in the bee intestine and not so much with species which are rarely found or which are incidental visitors. From the work of Bruce White (76) it is possible to differentiate the organisms that are normally present, at least in Scotland where his work was done. In this group of bacteria (*Bacillus constellatus*, *B. rigidus*, *B. influenzoides apis*, and *B. tenusi apis*), all four grow on media containing glucose and three on maltose media. None were reported to grow on media containing other sugars or on milk. *B. rigidus* grows feebly on potato while *B. tenusi apis* grows well on this medium. White (76, p. 76) concludes: "The organisms are as a whole characterized by their predilection for glucose-containing media and the fermentation of this sugar with-

<sup>4</sup> There is a large literature on the composition of pollen. For summaries of this work, see Parker (56) and Paton (57). The fact that certain pollens cause hay fever in susceptible persons has led to extensive studies of pollen, and the literature on hay fever may profitably be consulted.

<sup>5</sup> That bees do not need to ingest any protein during winter may perhaps be explained by the storage of protein material in the so-called fat body (42). It was at one time believed by some American beekeepers that dysentery in winter is caused by the consumption of pollen, and to remedy this condition it was advised that all combs containing pollen be removed from the hive during winter. It is now known that pollen consumption does not explain dysentery, and that bees are able to live through the winter without pollen (58). For those unfamiliar with the methods of beekeeping, it may be stated that when the pollen-bearing combs were removed, it became necessary for the beekeeper to provide additional carbohydrate food, which was done by feeding sugar sirup. This reduced or entirely eliminated the honey containing indigestible ingredients, thus preventing dysentery; whereas the beneficial results were attributed to the removal of pollen, which had nothing to do with dysentery. This removal of pollen had an indirect bearing on this trouble, however, for without pollen no brood could be reared, even though the temperature conditions became favorable for that activity; and the large addition to the feces, which would have resulted had brood rearing been carried on, was therefore prevented.

out gas production." The only organisms in the intestinal flora, which were found to grow on media containing lactose or sucrose, were inconstant species.

There are also yeasts and other fungi present in the intestine of the worker bee, but these are not a large factor in the flora. It would appear that because of their relative scarcity none of these fungi need be considered as likely to complicate the problem considered in this paper. In addition to pollen grains and microorganisms, there are present in the intestine hairs from the outside of the bee and various foreign bodies. Most of these probably play no part in hydrolizing the carbohydrates previous to absorption, but the presence of included material, such as pollen grains, may materially modify certain experimental results on the presence of enzymes, as will be seen later in an examination of the work of Petersen on starch digestion.

#### VITAMINS IN THE DIET OF HONEYBEES

Feeding experiments such as those recorded herein cannot be discussed without raising the question of the vitamin requirements of bees, a subject on which little is so far known. Honey, the normal carbohydrate food of bees, has been studied for its vitamin content (5, 20, 22, 26, 34, 69), and most workers find none. Pollen, on the contrary, has been tested only by Dutcher (22) who finds it effective in curing avian polyneuritis. It might be expected that pollen would contain at least vitamins A and B. The various microorganisms which occur in the alimentary tract of the adult bee may also be a source of vitamins, although none of them have been tested in this respect.

The question of the importance of vitamins to adult bees under normal conditions need not especially concern us in connection with the present problem. It is enough to know that under the conditions of the experiments and with the bees selected at random from the colonies, some of them survived for 30 days without obtaining food containing vitamins. Whether these bees were normal is not determined, and the only question at present discussed is whether or not they can utilize the carbohydrates given them, to which question they have given a definite answer. It is a fact of considerable importance and interest, however, that beekeepers consider it good beekeeping practice to give their bees a sirup of partially inverted cane sugar for the period of winter confinement, often extending over five months. Under the conditions within the hives during this time it seems that the bees survive and are in excellent condition for building up strong colonies in the spring on food free of vitamins. If honey does not contain vitamins, the bees, nevertheless, normally survive winters without vitamins in their food.

In spite of the number of workers who have tested honeys for vitamins, it is still perhaps safe to state that more work is needed. Honeys from various floral sources vary considerably in their chemical composition, flavors, and in many other distinguishable ways. It may therefore be that they vary in vitamin content. This is suggested by the marked variation in amber or yellow color, since vitamin A is associated with yellow carotin color at times. Carotin is one of the chief constituents of honey colors, and this suggests the desirability of conducting tests for vitamins with amber and dark honeys.

This is mentioned here with a realization that it doubtless has no bearing on the present problem. However, since the darker honeys, those containing more carotin, are known by beekeepers frequently to be the cause of dysentery, this in turn suggests that vitamins are unnecessary during the period of winter confinement to the hive, during which period brood is not normally reared.

#### ENZYMES IN HONEY

Considerable work (2, 3, 4, 18, 23, 24, 25, 27, 30, 44, 45, 46, 47, 52, 66, 75, 81) has been done on the presence of enzymes in honey, much of this work having been undertaken in an effort to find means for detecting adulteration. It is concluded that some of these enzymes have their origin in nectar (for example, catalase, according to Auzinger), and that others are produced by the bee and are added during the ripening process. This has a distinct bearing on the present problem, especially in view of the fact that several of the investigators of this subject report the presence of amylase in honey. This was reported first by Erlenmeyer and von Planta. Auzinger makes the definite statement, however, without any evidence to support it, that this enzyme is produced in the salivary glands. If this were the case, it would doubtless occur in the ventriculus of the bee and would serve for the hydrolysis of starch and dextrins (on the assumption that the enzyme present is the mixture of enzymes necessary for the complete hydrolysis of starch to glucose). In view of the fact that in these tests the pollen in honey is evidently not eliminated as a possible source of enzymes (Auzinger states definitely that the honeys were not filtered), and in view of the presence of amylase in pollens (57), it appears at least possible that the results reported are not fully trustworthy, in so far as these enzymes are assumed to be produced by the bees. That bees add invertase to nectar during the process of ripening is well known.

An enzyme to be functional in the processes of digestion of carbohydrates must be present in sufficient amount to act on the carbohydrates after they are ingested and must act so that the carbohydrate food, when there is but one present, may be hydrolysed fast enough to maintain life. This presumably means that no enzyme not produced by the bee itself will serve the purpose. To a considerable degree, therefore, the results obtained in the study of the enzymes in honey may be disregarded, at least in so far as the enzymes come from nectar. The detection of enzymes in honey by the use of glycerin and alcohol extracts should not be interpreted as meaning that the enzymes are produced by the salivary or digestive glands, as Auzinger seems to have done.

#### INVESTIGATIONS OF ENZYMES IN THE BEE

The physiology of the digestive processes in the honeybee has been investigated by Petersen (59), who reviews the essential physiological literature on this subject to the date of publication. The work which he did is quite unlike that herein recorded, and it is not necessary to discuss all his findings. On one point only is it necessary to examine his work in detail, and this will be done under the discussion of the utilization of starch. Biedermann (11) carefully reviews the literature on digestion of bees and other insects up to the time of publication.

While the present work was under way, there appeared an extensive paper by Pavlovsky and Zarin (58), which deals chiefly with the enzymes present in the various parts of the alimentary canal of the honeybee. The methods used by these authors were those usually employed in such work, namely, the preparation of enzyme extracts with glycerin or alcohol from the various organs to be studied. These authors record that they carefully washed out the included material (a precaution which Petersen does not record taking), which would presumably eliminate pollen as a source of enzymes to disturb their results. It is greatly to the credit of these investigators that they succeeded in getting out all the included material, for this must have been a difficult task. They report the presence of amylase (diastase) in the ventriculus. There are several places in the paper of Pavlovsky and Zarin in which their results are apparently stated incorrectly. For example, they state that they established the presence of lactase (58, p. 519) and later they state that they did not find lactase present (58, p. 527), while in the table summing up their results (58, p. 543) they record the presence of lactase in the stomach (ventriculus?). Sarin (82, 83) reports no lactase in the "stomach." Whether these errors were due to unfamiliarity with the language in which the paper appeared, which is not the native tongue of the authors, can not be learned. The paper reached the editor of the *Journal* in English and was not translated by him. The details wherein the results of these authors differ from those obtained in the present work will be mentioned under later headings in this paper.

#### CARBOHYDRATES AVAILABLE TO HONEYBEES

In order to understand the significance of the results obtained in the present work, it is desirable to review briefly the sources of the carbohydrates obtained in bees and the processes through which they pass in the formation of nectar within the plant, in the ripening of the nectar within the hive, and during digestion by the bee. Nectar, as obtained by the bees from floral and extra-floral nectaries, usually contains considerable sucrose, but there are small and varying amounts of glucose, levulose, and dextrins, together with the ash, water, coloring materials, and aromatic substances which are not considered in the present discussion. Some nectars probably contain no sucrose. The composition of various nectars has been discussed by various workers. (13, 17, 19, 68, 78.)

According to Bonnier the formation within the flower of a soluble food supply (sugar) as distinguished from starch is primarily that the flower may have a quickly available food reserve close at hand for the rapid development of the ovules after fertilization. It is only in certain plant species that an external secretion occurs, although the accumulation of sugar at the base of the flower occurs generally. The preliminary transformation of the insoluble starch (if the plant food supply is stored in that form) into soluble materials (usually sugars) is of interest here only in so far as the transformation from starch into sugars is sometimes incomplete, leaving a certain percentage of dextrins in the nectar. Since sucrose is not produced by hydrolysis of starch (38), the origin of this sugar, which in many cases is the chief constituent of nectar, is still somewhat in doubt. Since

sucrose and levulose may be produced directly by the action of chlorophyll, and as these are soluble materials, it is probable that the sucrose in nectar is produced directly by the chlorophyll and transported to the flower from the point of origin, and that it is not derived from insoluble storage foods. Invertase is generally found in plants and is doubtless involved in the formation of sucrose. Such a supposition would tend to explain the great variation in the composition of the secretion under varying external conditions, since in most plants sucrose is less abundant than is starch. The dextrin in nectar, however, is probably the result of the hydrolysis of starch, and the same may be true of the glucose. These points are of interest here only in so far as they suggest a reason for the increase in the percentage of dextrin under conditions when secretion of nectar is less rapid. It is well known to beekeepers that when secretion of nectar is slow, the honey from any given source is darker in color, and this is associated with an increase in the dextrin content of the nectar and consequently of the honey. The presence of dextrin in honey and honeydew honey indicates that it is not hydrolysed during the ripening process. There are also interesting variations in the color and abundance of nectar according to altitude, latitude, and other factors, some of which were pointed out years ago by Bonnier but which have become more clear with the great increase in beekeeping in the United States.

The production of nectar on the outside of the tissues of extra-floral nectaries is not a common occurrence at ordinary altitudes and latitudes. When such secretions occur the material exuded is as a rule higher in dextrin content than nectar from floral nectaries. Bonnier and Flahault (14) found that at high latitudes such external secretions are much more abundant than from the same plant species at points further south. A similar abundance of extra-floral secretion on the quaking aspen (*Populus tremuloides*) may be observed at high altitudes in the Rocky Mountains during the period of the growth of the leaves. Since extra-floral nectar secretion is greater at high altitudes and high latitudes, where the winter seasons are longer, it might be assumed that in such locations the quality of the food for the bees would be a serious problem. In fact, however, the food in such locations is usually of the best quality for winter use because of the abundant secretion from floral nectaries. It is well known that bees do not gather honeydews, either of plant or of insect origin, when there is abundant floral nectar.

Nectar or honeydew is collected in the honey stomach of the worker bee and brought to the hive. Here, after manipulation by the young bees inside the hive, it is deposited in cells of the comb, usually near the brood-rearing area. Only a small quantity is placed in each cell at first. By fanning their wings to create air currents, by raising the temperature within the hive especially near the brood nest, and perhaps by other activities of the bees as yet but slightly understood, the surplus water is evaporated. The remaining more concentrated solution (honey) is then brought together in the storage cells of the hive and is finally sealed over as fully ripened honey. During this ripening process a complete or almost complete inversion of the sucrose occurs, so that the final product, honey, contains but a small

amount of this sugar.<sup>6</sup> To this extent, therefore, the process preliminary to absorption occurs before the honey is actually taken by the bees as food. The enzyme necessary to the inversion of sucrose is present in all parts of the bodies of bees.

During a shortage of floral nectar bees may gather the excretions of aphids and other Hemiptera (insect honeydews) which they carry to the hive and manipulate just as they do nectar. There are few analyses of such material as it is gathered by the bees, but it is quite variable in water content, as can readily be seen when it occurs on leaves of trees. The ripening process is like that of floral nectar, but the final product (honeydew honey) differs from honey chiefly in the fact that the dextrin content is high. In the sample used in the feeding experiment recorded in this paper, the dextrin content was determined through the courtesy of the Bureau of Chemistry as 4.55 per cent. Another sample of honeydew honey received in 1916 by the Bureau of Entomology from Eagle Gorge, Wash., was found to contain 8.96 per cent dextrin. The usual dextrin content of normal honeys rarely exceeds 2 per cent, except in the most inferior honeys, and is less than 1 per cent in fine honey of the quality which beekeepers prefer to use for winter foods.

Other carbohydrates may also be found in honeydews or in honeydew honey after ripening. Unger (74) found mannite (a hexahydric alcohol) in considerable quantity on honeydew from *Juglans regia*, and Maquenne (51) found dulcite (a hexahydric alcohol) in honeydew from *Euonymus japonica*. As will be discussed later, melezitose (a trisaccharide) is found in certain honeydews. Because of the possibility of the presence of various carbohydrates in such compounds, it is desirable to determine the food value of many such substances to bees.

It may be well to point out that there is no suggestion that dextrins have any direct harmful effect on the bees. In the experiments carried out by Phillips and Demuth (63) on bees in a constant-temperature room (colony 3), the bees on honeydew honey stores lived but a short time. While they were undoubtedly nourished by the food provided them, the accumulation of feces stimulated them to great bodily activity and this activity in turn caused the temperature of the winter cluster to be raised. This should, however, not be interpreted as indicating that the accumulation of indigestible materials is in itself injurious; but the bees responded to this internal stimulus by increased muscular activity, which increased source of heat, raised the temperature of the cluster, and caused the bees to die of what the authors designated excessive heat production. No evidence was obtained that anything comparable to auto-intoxication occurs, although no examinations were made for microorganisms or other complicating factors which may have been present. The ease with which the condition known as dysentery may be relieved if the bees have opportunity for flight is against any such supposition.

It should also be pointed out that floral honey from any given species may differ greatly in the percentage of dextrin present. When honey is coming to the hive rapidly—that is when the flowers

<sup>6</sup> Not to exceed 8 per cent, according to the standard proclaimed Dec. 20, 1904, by the Secretary of Agriculture. There is considerable variation in the percentage of sucrose remaining in honeys from various floral sources, depending perhaps on the rapidity of the secretion and the consequent rapidity of the ripening process. Bonnier (18) states that mountain honeys (usually secreted rapidly) contain more sucrose than do those of lower elevations.

are secreting nectar freely—the resulting honey is lighter in color for the particular plant species than when the secretion is slow. This difference in color is associated with a difference in dextrin content, suggesting a more complete hydrolysis of insoluble carbohydrates to sugars within the plant or flower at the time when conditions are most favorable for nectar secretion.

It has been shown by numerous investigations that nectar secretion is more rapid, other things being equal, at high altitudes and latitudes. Honey of the North are almost all lighter in color than those at lower latitudes. Since in northern localities bees are confined to the hives without flight for long periods in winter, often over five months, it is clear that the quality of food is of first importance in the successful wintering of bees in those regions. It is extremely fortunate for the development of practical beekeeping that the honeys of the North are light in color and free of dextrans, for bees are able to live in confinement of such honeys for the full period of the winter.

On the other hand, the presence of a higher percentage of dextrans in so many of the honeys of the middle and southern latitudes of the United States seems to explain the fact that the winter losses are usually as high in these places as in the extreme northern parts of the country. Most of the honeys which are produced in late summer and autumn are dark in color, such as the asters and golden-rod of the lower latitudes, and these are about as poor for wintering as any floral honeys available.

Bees also obtain carbohydrate material from the pollens which they consume, most of this material being glucose, levulose, and sucrose. Some pollens are found to contain starch grains (72) and this is probably the only source from which the bees would normally obtain starch as an article of diet under normal conditions. The complex carbohydrates which compose the pollen shell, celluloses, and pectinases, are believed not to be utilized by bees but to be cast out with the feces after the internal material has been removed by digestive processes. It is also believed that the contents of the pollen grains can not be digested unless the pollen coat has been broken before ingestion.

In the making of candy for the shipping of queen bees with accompanying workers, a finely divided cane sugar is mixed thoroughly with honey of good quality. Before the composition of confectioners' sugar was so carefully guarded by the administration of the Food and Drugs Act of 1906, it frequently happened that a beekeeper would purchase a lot of such sugar which contained a considerable amount of starch, and when this was used the death rate of queen bees and workers to which it was fed in confinement was found to be much higher than when a pure cane sugar was used. This indicated that the presence of the starch is detrimental to the bees, which would hardly be the case if they contained or produced amylase as has been claimed.

Since the sugar used in these cases is not known to have contained any material other than starch and ground crystals of cane sugar, the trouble must have come solely from the starch, as cane sugar is readily utilized by the bees as food.

It will be seen from the foregoing summary that the problem of the availability of various carbohydrates as food for honeybees is one of

practical importance. No attempt has been made in this work to study the processes of digestion of the various carbohydrates, nor to analyze the feces of the bees on various foods. Such work would be exceedingly difficult because of the activity of the bees and especially because of the relatively short life of bees under experimental conditions. By restricting the food to a single carbohydrate it is possible to tell whether the bees are nourished by it. The work recorded is therefore not chemical in nature, but does indirectly throw light on the chemical processes which must occur within the alimentary canal of the bee.

### CONDITIONS OF THE EXPERIMENTS

Since bees kept in small wood and wire cloth cages in the laboratory are exceedingly active, due to the stimulus of light, the cages used in most of the experiments were kept in a cool, dark place in the cellar of the laboratory. Under these conditions the bees remained quiet, and because of this quietness doubtless lived much longer than they would in the light.<sup>7</sup> The cages used in the experiments were the same as those used by McIndoo (49) in his work on the olfactory sense of the honeybee. In the first two series of experiments about 100 honeybees were placed in each cage, while in the third series 200 bees were used. The third series of experiments were also modified by the fact that the bees were not placed in the cellar, but in a cool room in the laboratory where the light was not strong. The same methods were used in series 4 and 5 but no definite number of bees was used. Series 6 is discussed later.

In the first two series of experiments the bees used were chosen with great care by the following methods. The day previous to the beginning of the experiments the colony from which they were taken was moved carefully to a new stand in order that the field bees might return to their old location and that none of them would be included in the bees used in the feeding experiments. Then the bees used were hand picked to avoid very young bees which had not had a flight and which consequently have a considerable amount of feces in their rectums. By these means also the bees used were all about the same age. In the later experiments these precautions were not all taken, but the validity of the experiments were strengthened by the use of a larger number of bees. In all cases the field bees were removed by the moving of the colony, but in the later experiments a considerable number of bees were shaken into an empty box and were scooped up after being well mixed by shaking. In this way the handling of the bees was reduced and they were therefore subjected to less excitement and activity before the feeding experiments were begun. As previously stated, and as shown by the data presented, more bees were used in the later experiments, thus reducing the probable error of the averages.

The food and water provided were given in small vials inverted over the tops of the cages. These feeders were constantly moist but the contained solutions were held within the vials by atmospheric pressure. There was, of course, a slight loss of food from the fact

<sup>7</sup> That honeybees live longer when they have little work to do has long been known to beekeepers. It is quite outside the limits of the present discussion to give the evidence on this subject, but it is believed that bees are able to renew worn tissues to but a small degree, and that their term of life is shortened when they are called upon to perform muscular activity. The fact that during the larger part of their adult life bees use carbohydrates as food almost exclusively, perhaps explains this condition.

that some sugars crystallized out on the wire cloth and were not all used by the bees, but as it was not the primary purpose of the experiments to determine the exact amount of food consumed, this is considered an unessential detail. This crystallizing of the food was pronounced only in the case of melezitose.

The supply of food was renewed as necessary and counts were made of the dead bees daily, the dead bees being removed as counted. Except as noted, water was at all times available to all the bees, ordinary tap water being used for this purpose.

The honeybee is a cold-blooded animal (65), so that the temperature of the bees used in these experiments was the same as or only very slightly higher than that of the air surrounding them. The action of enzymes increases rapidly with a rise in temperature and is usually at its maximum at temperatures of 37° to 40° C. It may then be assumed that under the conditions of the experiments the enzyme action was reduced below that which would occur within the hive at a temperature of about 35° C. Since bees are able to live for long periods in winter at temperatures varying between 14° and 20° C. without injury, it is safe to assume that the temperatures encountered in these experiments were in no way preventive of enzyme actions of an essential character.

#### FIRST SERIES OF EXPERIMENTS

Fourteen cages with about 100 worker bees in each cage were prepared for feeding, the preparation of the cages and the placing of the food and water vials having been completed at 4 p. m. At the time when the cages were filled, many of the older bees were out of the hive and the younger bees taken tended to live longer than the average of the bees of the colony. So far as could be seen, there was no difference in the bees of the several cages. Beginning the next day and on each succeeding day the dead bees were removed from each cage at 4 p. m. and counted, as recorded in Table 1. Doubtless all these bees carried with them a certain amount of food when they were put into the cages. This food and their reserve apparently were sufficient to prevent death for an average of 1.74 days in the lot of bees to which no food was given, the last bee dying on the third day. It is therefore proper to conclude that when the bees lived more than three days or longer than an average of 1.74 days within the limits of the probable errors, they were able to utilize as food the material given them. Because of different conditions of temperature, the average length of life of the bees without food in the different series is unlike, and therefore it is undesirable to make detailed comparisons between different series.

The following plan was used in feeding the bees in the first series:  
*Lot No. 1.*—No food given. Water at all times available (control cage).

*Lot No. 2.*—Glucose, 50 per cent solution.

*Lot No. 3.*—Levulose, 50 per cent solution.

*Lot No. 4.*—Sucrose (C. P.), 50 per cent solution.

*Lot No. 5.*—Honey from the apiary of the Bureau of Entomology, probably chiefly from tulip trees. This honey was dark in color and doubtless contained relatively large amounts of dextrin. No analysis was made. The percentage of dextrin was smaller than in the case of the honeydew honey used (lot 12).

*Lot No. 6.*—Lactose, 1 part in 10 parts of water.

*Lot No. 7.*—Raffinose, 1 part in 2 parts of water.

*Lot No. 8.*—Mannose, 50 per cent solution.

*Lot No. 9.*—Maltose, 50 per cent solution.

*Lot No. 10.*—Dextrin 1 part, sucrose (commercial cane sugar), 1 part, water 2 parts. Compare use of dextrin in the second series.

*Lot No. 11.*—Starch (potato) 1 part, sucrose (commercial cane sugar) 10 parts, water 10 parts. Compare use of starch in other series.

*Lot No. 12.*—Honeydew honey. This was collected in the apiary of the Bureau of Entomology then at College Park, Md., in 1909, removed from the combs by heating after crystallizing hard, and was then stored in tin cans. Because of the fact that this honeydew honey crystallized so hard, there is now reason to suspect that it contained melezitose. Since this trisaccharide is satisfactory as food for honeybees (see series 3), this possibility makes no special difference in the results of this test. Unfortunately by the time the presence of this sugar was suspected in honeydews gathered near Washington, all of the material collected in 1909 had been used.

*Lot No. 13.*—Brown sugar, commercial. No analysis was made. The purpose of using this material was to determine if possible whether the beekeeper is safe in using it as winter food for his bees. Under such circumstances the bees would be confined to the hive longer than in this series of feedings, and the high percentage of nonsugars would doubtless cause dysentery. A 50 per cent solution was used.

*Lot No. 14.*—Commercial glucose.

Table 1 shows the daily counts of dead bees in each cage, the average length of life, and the probable error of the length of life of these bees. Records were kept of the solid food consumed when food was given and in each case it was found that considerable quantities per bee per day were ingested. These records are not here given as the amounts are unimportant in the present discussion.

TABLE 1.—Daily death rate and average length of life of bees on various foods, first series

Food	Number of deaths after different periods (days)																	Total number of deaths	Average length of life (days)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
No food.....	32	62	6															100	1.7400±0.0677
Glucose.....	0	3	8	2	9	5	11	9	12	10	9	15	5	1				99	8.3656±0.2125
Levulose.....	1	2	5	5	10	5	11	6	4	6	9	17	9	5	2	1		98	8.9160±0.2188
Sucrose.....	0	7	1	14	9	10	8	10	8	13	10	5	4	0	2			101	7.0040±0.2156
Honey.....	20	0	0	4	2	12	2	3	6	8	4	6	11	10	9	3	1	101	8.8020±0.3583
Lactose.....	45	51	1	1	1	1												100	1.6500±0.0534
Maltose.....	35	57	3	5														100	1.7800±0.0492
Mannose.....	4	14	30	41	8	3												100	3.4400±0.0709
Maltose.....	5	8	6	17	18	8	7	6	2	4	5	9	4	4	1	1		100	6.9200±0.2545
Dextrin, sucrose.....	6	7	13	16	14	12	11	3	10	6	2	2						100	5.3800±0.1756
Starch, sucrose.....	1	3	2	6	6	3	7	7	4	11	7	16	11	5	7	1	2	101	9.8713±0.2589
Honeydew.....	10	0	5	7	9	12	13	10	11	21	8							100	7.2000±0.2017
Brown sugar.....	2	15	12	13	9	12	22	10	2	3								100	5.1000±0.1540
Commercial glucose.....	5	7	17	25	29	12	3	3	1									102	4.3235±0.1050
Temperature, °C.....	23	23.0	24	23.5	23.4	23.4	20.6	20	19.5	21.2	22.1	21.2	21.6	22	22.8	23	20.6		

## SECOND SERIES OF EXPERIMENTS

A second lot of cages of the same kind were filled with bees and again fed in the same way, the differences in food being noted later. In this series, the temperature conditions were somewhat better for longer life without food, as the bees were less active. There may have been slight differences in the amount of food carried by the bees when put into the cages, but this could not be determined. Attention should be called to the rather heavy death rate on the fourth and fifth days from unexplained causes, which tend to reduce the average length of life in this series. In spite of this the average and actual time of the death of the last bees were larger than in the first series. Counts of dead bees were again made daily.

Except as noted the foods given were as in the first series. The following feeding plan was used:

*Lot No. 1*—No food given (control).

*Lot No. 2*—Glucose.

*Lot No. 3*—Levulose.

*Lot No. 4*—Sucrose.

*Lot No. 5*—Honey.

*Lot No. 6*—Lactose.

*Lot No. 7*—Raffinose.

*Lot No. 8*—Mannose.

*Lot No. 9*—Maltose.

*Lot No. 10*—Dextrin, 50 per cent solution. (No sucrose added).

*Lot No. 11*—Starch. One part potato starch was heated in four parts of water at a temperature below boiling, the water content corrected, and the solution given the bees. The cooking may have changed a slight amount of the starch into dextrins and dextrose but most of it remained as starch. The purpose of the heating was to break up the starch grains and to make the food more easily ingestible.

*Lot No. 12*—Honeydew honey.

*Lot No. 13*—Brown sugar.

*Lot No. 14*—Commercial glucose.

Table 2 shows the results of the second series of feeding experiments. Except for changes in the foods given, these results may be compared with the data given in Table 1 covering the availability of the foods, but necessary allowance must be made for differences in temperature and other factors which caused slower death rates in the second series.

TABLE 2.—Daily death rate and average length of life of bees on various foods, second series

Food	Number of deaths after different periods (days)																						Total number of deaths	Average length of life (days)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
No food	0	2	18	36	33	10	1	14	6	8	6	2											100	4.3400±0.0662
Glucose	1	1	0	28	15	6	4	5	1	3	4	5	5	0	1	4	0	1					100	6.1700±0.1797
Levulose	0	0	10	26	21	8	6	6	4	3	3	3	3	4	1	3	0	1					100	6.7400±0.2518
Sucrose	1	2	4	28	19	11	3	4	2	4	3	4	4	6	1	1	0	1					101	6.980±0.2937
Honey	1	0	4	28	12	6	2	5	4	9	6	6	8	2	3	2	2						100	7.8200±0.2720
Lactose	5	14	15	48	11	6	1	1															101	3.7200±0.0866
Raffinose	0	3	13	28	36	18	2																100	4.5400±0.0727
Mannose	0	0	18	20	38	11	4	4	7	1	0	5	6	3	2	2	1	0	1	0	0	1	100	4.5400±0.0697
Maltose	0	1	6	33	19	4																	100	4.5400±0.2850
Dextrin	0	7	28	29	27	8																	100	4.010±0.0731
Starch	1	11	18	36	22	9	3																99	4.000±0.0812
Honeydew	0	1	40	19	20	11	3	2	4	1													100	4.4237±0.1127
Brown sugar	0	0	18	43	19	10	5	3	2														100	4.3800±0.0921
Commercial glucose	0	2	20	44	11	7	4	6	1	3													98	4.6122±0.1183
Temperature, °C.	17.4	17.4	20	19	10.4	20	20	21.4	19.6	17.6	16.6	16.1	15.1	14.5	15	15.1	15	15					17.4	

## THIRD SERIES OF EXPERIMENTS

The finding of the rare trisaccharide melezitose in honeydew honey collected in Pennsylvania in 1917 (37) led to a further search for this sugar in honeydew honeys. It was found that this sugar is occasionally gathered from coniferous trees near the apiary of the Bureau of Entomology in considerable quantities. A supply of purified melezitose was obtained for feeding experiments from the Bureau of Chemistry to determine whether honeybees are able to utilize this sugar as food.

Three lots of bees, each of 200 individuals, were placed in cages like those used in previous experiments September 29. One lot was given no food, one was fed on sucrose (commercial cane sugar, 50 per cent solution), and the third was given a 50 per cent solution of melezitose. Water was provided each lot of bees as in previous experiments. The bees were placed in the cages in the afternoon of September 29, and the dead bees were removed and counted daily as in previous experiments. In this instance the cages were kept in a room in the laboratory with diffused but without direct sunlight. It was necessary for the investigators to leave the laboratory October 28 for a considerable period, and it was therefore impossible to continue the counts after that date. The results covering the availability of melezitose as food were entirely conclusive, but the figures given can not be compared with those in other series. Fifteen bees still remained alive in the cage given cane sugar and one bee remained in the melezitose cage at the close of observations. Table 3 gives the data obtained.

It was noticed that the melezitose tended to crystalize about the feeder, and to remedy this the water bottle was shifted to the places where the crystals appeared, so that they might be dissolved and used by the bees. Throughout the experiment the bees fed on cane sugar were noticeably more active than those fed on melezitose, and this activity possibly hastened their death. The bees on cane sugar died at the average rate of 6.38 per day while those on melezitose died at the average rate of 6.86 per day. During the first 15 days 84 bees died on cane sugar and 140 on melezitose, or at the average rates of 5.60 and 9.33 daily, respectively. Between the twelfth and fifteenth days, during which no counts were made, an unexplained heavy death rate occurred in both cages.

TABLE 3.—Daily death rate of bees on various foods, third series

Food	Number of deaths after different periods (days)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
No food.....	125	75													
Cane sugar.....	5	0	1	0	0	(1)	2	8	0	8	11	12	(1)	(1)	37
Melezitose.....	10	3	0	1	4	(1)	21	8	5	16	23	24	(1)	(1)	25

Food	Number of deaths after different periods (days)																
	16	17	18	19	20	21	22	23	24	25	26	27	28	29			
Cane sugar.....	8	7	12	6	(1)	16	2	8	(1)	6	9	(1)	14	3			
Melezitose.....	2	10	12	4	(1)	6	4	1	(1)	10	1	(1)	4	15			

<sup>1</sup> Dead bees not removed and counted.

## FOURTH SERIES OF EXPERIMENTS

In order to extend these observations to other carbohydrates, a fourth series of feedings was undertaken. In general the methods were the same as for the previously reported series, except that the bees were not hand picked and a larger number of bees was used. There were some drones in the cages, but the number was small and these are omitted in the calculations. The bees were placed in the cages in the early afternoon, the operation being completed at 2.30 p. m., at which time the various foods were put into place. Cages 2 and 3 of this series contained bees without food, kept in other places to determine what effect increased activity has on the length of life of worker bees without food. This has been reported previously (60) and is here omitted.

The following foods were given the different lots in this series:

*Lot No. 1.*—No food given. Water at all times available.

*Lot No. 4.*—Glycerin, undiluted.

*Lot No. 5.*—Dextrin, 50 per cent solution.

*Lot No. 6.*—Inulin to which water was added without heat.

*Lot No. 7.*—Soluble starch in two volumes of water.

*Lot No. 8.*—Sucrose, 50 per cent solution.

*Lot No. 9.*—Galactose in 4 volumes of water.

*Lot No. 10.*—Lactose in 10 volumes of water.

*Lot No. 11.*—Raffinose in  $2\frac{1}{2}$  volumes of water.

The varying amounts of water in the food was necessitated by the comparative solubilities of the various substances. In such cases as soluble starch, for example, the material was not in actual solution, but the water present served to carry the food to the bees, and other water was available in all cages.

With the possible exception of glycerin the bees took carbohydrate when it was offered them. In the case of the glycerin, the humidity of the cellar in which the bees were kept may have caused moisture to be absorbed, and the volume of food had not noticeably decreased at the time the last bees died. Table 4 gives the results of the fourth series of experiments.

TABLE 4.—Daily death rate and average length of life of worker bees on various foods, fourth series

	Number of deaths after different periods (days)																	Total number of deaths	Average length of life (days)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
No food.....	42	141	87	4														274	2.1034±0.0286
Glycerin.....	75	148	91	4														318	2.0755±0.0285
Dextrin.....	61	98	81	7														247	2.1377±0.0350
Inulin.....	79	181	51	1														312	1.9167±0.0248
Soluble starch.....	95	102	65															322	1.9068±0.0253
Sucrose.....	40	15	1	7	11	20	9	17	20	13	15	5	4	10	2	2	1	102	6.0719±0.2090
Galactose.....	63	93	66	4														226	2.0487±0.0359
Lactose.....	64	113	116	8	1	1												303	2.2475±0.0321
Raffinose.....	64	73	143	21														301	2.4020±0.0349
Temperature ° C.....	20.4	19.8	18.8	18.8	19.4	19.0	18.8	18.8	18.8	22.0	19.2	19.8	19.4	20.6	19.8	19.2	21.5		

## FIFTH SERIES OF EXPERIMENTS

In this series a number of carbohydrates were introduced which probably never occur in the food of bees, but by means of which the presence of certain enzymes in the alimentary tract may be tested. These will be discussed later, but special reference may here be made to the use of trehalose. In this series one control cage of bees was given water while another cage received neither food nor water. Daily counts were made as in other series and in every case there was reason to believe that solid carbohydrate materials were actually ingested by the bees. It should be noted that if the bees had been able to hydrolyze some of these carbohydrates a death rate even more rapid than that of starved bees might have been expected, since when split these glucosides become poisonous. No such change in death rate was observed. Cages 1 and 2 of this series contained drones only and are not here discussed, as they have been reported elsewhere (61).

The following food materials were tested in this series of experiments:

*Lot No. 3.*—Sucrose, in 2 volumes of water; no other water given.

*Lot No. 4.*—Amygdalin, in 2 volumes of water; no other water given.

*Lot No. 5.*—Salicin, in  $2\frac{1}{2}$  volumes of water; no other water given.

*Lot No. 6.*—Mannite in  $2\frac{1}{2}$  volumes of water; no other water given.

*Lot No. 7.*—Xylose, in 2 volumes of water; no other water given.

*Lot No. 8.*—Mannose in 2 volumes of water; no other water given.

*Lot No. 9.*—Rhamnose in 2 volumes of water; no other water given.

*Lot No. 10.*—Trehalose, in 2 volumes of water. At the beginning of this feeding 10 gm. of dry trehalose were available and by the third day half of this quantity had been taken by the bees and they were apparently living well on it. On the eighth day the last drop of food was removed from the feeder and 5 c. c. of water was placed in it to wash out any food that might remain. No other water was given. Whatever food the bees had from the eighth day until the last bees died must have been carried in their honey stomachs. The average length of life is therefore not fully indicative of the ability of the bees to utilize this sugar.

*Lot No. 11.*—*d*-Arabinose, in two volumes of water, no other water given.

*Lot No. 12.*—*l*-Arabinose, in two volumes of water, no other water being given.

*Lot No. 13.*—No food (control), water given in a feeder.

*Lot No. 14.*—No food, no water given.

Table 5 shows the daily death rate and the average length of life of the bees in this series of experiments, and the probable error of the average. Because of the unusual character of some of the materials offered to the bees in this series, it seemed desirable to force the several carbohydrates on them by providing no water other than that in which the carbohydrates were dissolved. In every case material was removed from the feeder bottles, indicating that carbohydrates were actually ingested. It may safely be assumed that at least some of these materials were not at all attractive to the bees, but since some of the material was ingested in each case, it may further be assumed that if it had been hydrolyzed the length of life would have been modified either by hastening death or by prolonging life. The rather

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surprising result from the use of trehalose will be discussed later. The absence of water from cage 14 seemed to make no appreciable difference in the length of life of bees without food, since the average length of life in cages from which water was withheld was actually slightly longer than for bees without food to which water was supplied. This difference is of course too small to have any significance, since the average length of life of bees in several cages that starved in spite of the fact that they took carbohydrates, slightly exceeded the average length of life for bees without food and without water.

TABLE 5.—Daily death rate and average length of life of worker bees on various foods, fifth series

	Number of deaths after different periods (days)																			Total number of deaths	Average length of life (days)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
Sucrose	5	3	17	8	20	9	34	22	47	41	38	24	23	16	6	1				320	8.1750±0.1387
Amorph. chitin	10	78	171	83	1															233	2.7850±0.0274
Saltin	31	75	171	17																234	2.5918±0.0296
Ammonite	13	62	141	18	4															348	3.1839±0.0350
Xylose	35	61	174	19																322	2.4232±0.0244
Rhamnose	32	101	170	15																383	2.1933±0.0665
Arabinose	70	134	176	18																361	2.8463±0.0917
Trehalose 1	24	8	13	25	31	32	30	10	24	37	30	14	31	15	9	2	1			336	8.0988±0.1415
Trehalose 2	34	52	168	41	1															296	2.7390±0.0330
Arabinose	22	89	181	34	1															327	2.7034±0.0281
No food	24	175	175	3																377	2.4164±0.0216
No food, no water	14	90	226	42																372	2.7957±0.0238

<sup>1</sup> See explanation in text of the food supply on this sugar.

## SIXTH SERIES OF EXPERIMENTS

Two lots of bees were placed in the small cages November 24, one being provided with a paste made of white clover honey and soluble starch (121 bees) and the other with a paste made of soluble starch and cane sugar (169 bees). Both lots of bees were given water in a small feeder. The bees were kept in a laboratory room with subdued light at ordinary room temperature. The purpose of these feedings was to follow the starch through the alimentary canal and not to establish the death rate. The bees on honey and starch died somewhat faster than those on starch and sugar, probably because they were able to take more food and thus to clog their alimentary tracts more rapidly. The last bee living in the cage with sugar and starch was removed alive December 4, while the last bee on honey and starch was removed November 29. The paste of sugar and starch tended to glaze, so that the bees could not readily take the food. To overcome this difficulty a drop of water was placed on the paste from time to time. The bees immediately took the food more readily. The details of the examinations of this series will be treated under the discussion of starch. The "soluble starch" used was tested before using and every grain examined stained deep blue with iodine.

## THE AVAILABILITY OF THE CARBOHYDRATES AS FOOD FOR HONEYBEES

From the foregoing records, it will be seen that in certain cases the bees died as quickly after ingesting certain carbohydrates as they did when given no food. This fact is interpreted to indicate that these carbohydrates are unavailable as food, even though actually ingested. In other cases length of life was materially increased by the eating of carbohydrates, which is interpreted to mean that such carbohydrates serve as food for the bees and are partially, and probably in most cases almost entirely, utilized. The results of experiments in which mixed foods were given indicate that not all components of the material can be utilized.

The materials used in these experiments vary greatly in taste to the human tongue. That a carbohydrate tastes sweet to man is not proof that this is true for bees, and some differences in taste to the bees may have made an appreciable difference in the amount of material ingested and may therefore have influenced the length of life. In some cases the bees took very small amounts of the material available to them and may actually have starved. In all cases they took some of the material offered (except possibly glycerin), and unless the carbohydrate served as food they died as if no food were given.

In certain cases, such as the glucosides, the material was offered the bees as a means of determining whether they are able to hydrolyze it. In such cases it is assumed that if hydrolyzation were accomplished the length of life would actually be decreased rather than increased, since the products of the splitting of these materials produces a material known to be poisonous to insects.

A summary of the results of these tests follows:

## RESULTS OF EXPERIMENTS

### BEES WITHOUT FOOD

The controls for these tests were bees which had no food other than that which they carried in their honey stomachs at the time they were placed in the cages. In the second series the bees lived without food for 4.34 days,<sup>8</sup> while in the first series the average length of life was only 1.74 days. The difference in temperature doubtless accounts for such variation. Later experiments gave still different results, but in no other case did the starved bees live as long as in the second series of feeding experiments. For this reason, the results of all feedings in the second series are less definite than those in other experiments.

In colonies wintered in cellars with proper temperature conditions bees have been known to live for several weeks when separated from their food supply. Under the conditions surrounding the bees in these tests however, an average length of life of 4.34 days seems high. In other experiments (60) it has been found that high temperatures and increased activity greatly increase the death rate. In certain cases it was found that some bees actually died of starvation within a few hours after being put into cages, the food in the alimentary tract being wholly exhausted. In other experiments not here recorded, the quantity of food consumed by bees hour by hour was determined by weighing, and an astonishing quantity was found to be consumed when the bees were at all excited, and their metabolic activity thereby increased.

In considering the availability of each carbohydrate used it is necessary to compare the average length of life (and to some extent the greatest length of life observed) with the average length of life of the bees without food in the same series of feedings, since in each feeding series the various lots were subjected to the same external conditions, so far as known.

### GLUCOSE

The bees lived well on this sugar as would be expected from the fact that it is one of the chief constituents of honey. The results of the second feeding were less conclusive than those of the first feeding, but the differences in these results are insufficient to suggest any difficulty with glucose as a food. Glucose being a simple aldohexose sugar, no enzyme is required to make it available for absorption. It is evidently assimilated readily.

### LEVULOSE

As would be expected, this sugar served as food for the bees in both feedings (series 1 and 2). In the first feeding the bees lived as long as those fed on honey, within the limits of the probable error, and only a slightly shorter time than those on the cane sugar and starch mixture. In the second feeding they lived slightly less time than those on honey and as long as those on maltose, within the limits of the probable error. This being a simple ketohexose sugar, no enzyme is required to render it available for absorption. It is evidently readily assimilated.

<sup>8</sup> In the discussions probable error figures are omitted but they will be found in the tables for each series of feedings.

## GELACTOSE

This sugar was fed only in the fourth series. The bees were not nourished by it and died as rapidly as the bees without food, within the limits of the probable error. This is a simple sugar and no enzyme is needed to render it available for absorption, so that apparently it is unsuitable for assimilation by honeybees. This simple aldohexose sugar occurs as a constituent of lactose and of raffinose and in more complex compounds, and attention may be directed to the unavailability of lactose as food for bees (p. 25).

## MANNOSE

This simple aldohexose sugar does not occur in nature in simple form and therefore would not be expected normally to form part of the food of bees. It was tested in series 1, 2, and 5. In series 1 the bees on this food lived 1.977 times as long as did the bees without food, or 15.65 times the sum of the probable errors. In the second series they lived 1.046 times as long as the bees without food, or 1.47 times the sum of the probable errors, while in the fifth series the mannose bees lived 1.214 times as long as the starved bees, or 8.915 times the sum of the probable errors. In the first series the last bee on mannose lived twice as long as the last bee without food; in the second series the last bees of the two lots died the same day; and in the fifth series the last bee on mannose lived one day longer than the last bee without food.

The mannose used was  $\alpha$ -dextro-mannose. No enzyme would be required to split this sugar so as to make it available for absorption, and although on the whole the bees on this food were able to live somewhat longer than starved bees (in all cases in which the length of life was greater than the sum of the probable errors and in two cases in which it was several times the sum of the probable errors) it still appears not to be readily available for absorption and assimilation. So far as the author has been able to learn, this sugar has not been tested with other species of animals. Mannite, an alcohol, will be discussed later.

## SUCROSE

The first disaccharide to be considered is sucrose, which occurs widely in nature, as in cane and beet sugars and in nectar. This sugar was used in all of the first five series of feedings, serving as a control for the other carbohydrates used. The bees lived well on it in all cases.

Cane sugar is used extensively by beekeepers to supply their bees when there is a shortage of honey, and is used especially for winter food when the honey in the hive is of inferior quality (indicating a high percentage of dextrins). It is well known, therefore, that bees are able to use this sugar as food. Since it is often maintained, however, that bees need the salts and other materials in honey which are lacking in highly refined cane sugar, the only question which ever arises in the minds of beekeepers is whether cane sugar is as good as honey for use in the spring when brood is being reared abundantly. There is nothing in the results of these experiments to throw any light on this subject.

By the action of the enzyme invertase (sucrase) each molecule of sucrose is hydrolyzed to form one molecule each of glucose and levulose, both of which, as has been seen, are available for assimilation

by bees. In feeding sugar sirup, beekeepers often partially invert the sugar by the use of an organic acid (usually tartaric) and heat. The purpose of this partial inversion, however, is not to make the sugar assimilable but to prevent the formation of crystals which the bees can not use without waste. As the result of partial inversion of the sugar before feeding, followed by still further inversion from the manipulation of the sirup by the bees during storage, a balanced supersaturated sirup is formed which will remain liquid for months.

Invertase is exceedingly abundant in nature, being found in many species of plants and animals. That this enzyme is present in bees has been known for many years (4, 58, 59, 67, 82, 83). Several years ago Doctor Hudson, formerly in charge of the carbohydrate laboratory of the Bureau of Chemistry, was able to extract invertase from all parts of bees that had been dead for some time, and the amount of the enzyme present was found to be large (unpublished data). Invertase is also present in many pollens (31, 39, 57), but probably not in all. Invertase is also present in honeys (23, 24, 25, 46, 59). This is indicated by an interesting beekeeping practice recommended years ago by Doolittle, of putting unheated honey in sugar sirup to be used for winter food "to prevent granulation."

According to Hudson and Paine (36), invertase fails to act in alkaline solution and rises to a maximum in weakly acid ones. The PH value of the normal ventriculus of the worker bee is 6.8 to 7 (neutral). It is about the same for the rectal ampulla, except when the latter is heavily loaded with pollen, in which case the PH value may be as low as 6.4.<sup>9</sup>

#### LACTOSE

This disaccharide sugar is a constituent of milk and by the action of the enzyme lactase is hydrolyzed into the monosaccharide sugars, galactose and glucose. It was tested in series, 1, 2, and 4. In each case the bees died as if starved, and in two cases sooner. It will be seen from the discussion of galactose that bees are not nourished by that sugar. If lactose were hydrolyzed by bees, there would be reason to expect that the glucose constituent would serve as food for them, regardless of whether or not the galactose is utilized. Lactase is also from its composition a  $\beta$ -galactoside (70) and compounds of this group are hydrolyzed by emulsin, which is sometimes considered as consisting of a mixture of at least four distinct specific enzymes (6). The failure of the bees to utilize lactose therefore suggests the absence of emulsin, as is also indicated in other tests with glucosides.

In all tests with lactose the bees died as soon as if they had been given no food, so that they are evidently not nourished by it. In the first series of feedings they took almost no lactose, but they did take somewhat more in the second. Some lactose was taken in the fourth series, but the quantity was not weighed. Because of the low solubility of lactose, it was necessary to make a dilute solution. The results indicate, however, that this was not the cause of the death of the bees.

#### MALTOSE

In both feedings (series 1 and 2) the bees lived considerably longer, 3.977 and 1.622 times, respectively, on maltose than did the bees without food. In the first feeding they lived as long as did the bees

<sup>9</sup> Determination made by A. P. Sturtevant.

on honeydew honey, within the limits of the probable errors, and in the second feeding they lived slightly longer. They are evidently able to utilize this carbohydrate as food.

Maltose is a stage in the transformation of the insoluble and highly complex polysaccharide starch into the simple monosaccharide glucose. This action in nature is brought about by the action of enzymes of the amylase group, but it is usually believed that there are several distinct enzymes, each one of which is limited in its action to a particular stage of the series of changes which occur. The original action is variously described as either the formation of dextrin and maltose, or of dextrans alone. If dextrans are formed then there is a second stage in the action in which maltose is formed. After maltose (a disaccharide sugar) is once formed, by whatever steps, it is further hydrolyzed by the action of maltase (glucose) an enzyme of the invertase group, each molecule of maltose forming two molecules of glucose. The enzymes of the amylase group are not concerned in this action. The enzyme necessary for the splitting of maltose (maltase) is evidently present in bees.

The results obtained in feeding maltose are especially interesting in view of the inability of bees to utilize dextrans and starch, as will be later shown. Maltose is the first step in the transformation of starch into dextrose which the honeybee seems to be able to utilize as food. There was doubtless a small quantity of dextrin in the material fed but this, as will be seen later, is unavailable to bees.

#### TREHALOSE

This is a disaccharide sugar which is hydrolyzed into two molecules of glucose. It does not reduce Fehling's solution and is one of the most difficult sugars to hydrolyze with acids. It is not acted upon by the enzymes maltase, invertase, emulsin, or amylase but requires a specific enzyme, trehalase. Because of the fact that the bees were able to utilize this sugar in series 5, it seems necessary to discuss the distribution and splitting of this rare sugar at some length.

This sugar was first noted by Wiggers (77)<sup>10</sup> in 1832, when he observed crystals from the water extract of ergot on rye, which he called Mutterkornzucker. It was differentiated from other sugars by Mitscherlich (54) in 1856, who gave it the name mycose. He determined the formula and described the crystal form. In 1858 Guibourt (32, 33) described the trehala manna produced by the weevil *Larinus nidificans* on the twigs of a plant of the genus *Echinops* in Persia.<sup>11</sup> The material which Guibourt studied was turned over to Berthelot (1857-1859) (7, 8, 9), who extracted from it a sugar which he named trehalose, the name still commonly used, although it has since been determined that it is identical with the sugar seen by Wiggers (77) and Mitscherlich (54). Beginning in 1889, Bourquelot published a large series of papers on the finding of trehalose in various fungi, among which it is widely distributed during the period of rapid growth. The sugar disappears rapidly after the picking of the fungi and is replaced by mannite. It also occurs in myxomycetes, in ferns, in certain marine algae, and in the

<sup>10</sup> This reference and some others which from their nature were not consulted by the author, are taken from an unpublished manuscript which the author was privileged to consult. HARDING, T. S. BIBLIOGRAPHY CONCERNING TREHALOSE. [Unpublished manuscript in U. S. Dept. Agr., Bur. Chem. Carbohydrate Lab.]

<sup>11</sup> Regarding this insect and its product, see (64).

resurrection plant, *Selaginella lepidophylla*, of Mexico and western Texas. A sugar, iso-trehalose, has been artificially produced by Fischer and Delbrück (29).

This list of the known sources of trehalose has been given to show the improbability that this sugar has ever served as a natural food for honeybees. Apparently trehalose replaces sucrose in those plants which contain no chlorophyll and which do not manufacture starch. It serves as a reserve food material which can be utilized by these plants only after hydrolysis of the sugar into glucose. It is, of course, found in some green plants, as has been mentioned, but it has so far not been found in any plant from which honeybees make collections of sugar in the form of nectar. It is probable that other sources of trehalose may be discovered.

The inversion of trehalose into two molecules of dextrose was first described by Bourquelot. He found that trehalose was not affected by the invertase of brewer's yeast or by emulsin. With maltase he encountered some difficulties, for the enzyme obtained from *Aspergillus* split both maltose and trehalose. By the application of heat he was able to show that the ferment ceased to act on trehalose at 64° C., while it continued to act on maltose up to 74°, thus proving that two separate enzymes are present. It appears from Bourquelot's work, therefore, that for the splitting of trehalose by natural means a specific enzyme, trehalase, is required. It is important to note that trehalose, unlike sucrose, is split only with difficulty by the use of dilute mineral acids (50, 79, 80), and for the complete splitting into glucose only two hours' heating at 110° in the presence of 5 per cent sulphuric acid is necessary. It is not to be believed that the splitting which evidently occurred in the utilization of this sugar by the honeybee is due to the presence of acids in the alimentary tract, as appears to be the case in the splitting of inulin in man. Fischer (28) questions Bourquelot's conclusion of the necessity of a specific enzyme, since when one part of trehalose was kept at 33° for 40 hours with 5 parts of dry pure yeast and 10 parts of water containing 2 per cent thymol, 20 per cent of the reduced sugar was formed. He also found that trehalose is hydrolyzed by amylase, but because of the uncertainty which still surrounds the various enzymes, Fischer makes no positive statements regarding these facts.

In considering the results of Fischer, Bourquelot and Gley (15, 16) published two small papers giving data that appear to be conclusive proof that a specific enzyme is required for the splitting of trehalose. They found that although dog-blood serum and human urine contain a very active diastase, as they showed for the particular materials used in their experiments, those substances are without action on trehalose, and therefore the diastase of malt is a mixture of enzymes containing both diastase and trehalase. They also found later that beef-blood serum was without action on trehalose. In their later experiments they found that trehalose is hydrolyzed in the intestine of the adult rabbit, but that there was no reduction from the action of the pancreas. Extracts of pancreas and intestine of a young dog gave negative results.

Before the bees were fed trehalose, its utilization unfortunately was not predicted and an inadequate amount of the sugar was obtained. Only 10 gm. were available. As a result, the average length of life is not found to be quite so great as in the case of the

bees fed sucrose during the same series of feedings. Before death occurred for all the bees, however, the supply of trehalose was entirely exhausted, and the last bees doubtless died of starvation. In the notes made at various times during the course of the experiment, it is recorded that the bees were as active as those on sucrose. Eight days after the first feeding, when the supply of trehalose sirup in the feeders was exhausted, the bees were in fine condition, and the last bee did not die until 17 days after the first feeding. The last bee on sucrose did not die until two days later, but this lot of bees took more food (because it was available), and this is doubtless the explanation of the longer life of the sucrose-fed bees. It appears from the records of this experiment that the bees on trehalose lived as well on this sugar as did the bees on sucrose. The contrast in series 5 with the bees on various foods that are not utilized and with those to which no feed was given is most striking.

It appears, therefore, that whatever enzyme is necessary for the splitting of trehalose, that enzyme is found in the alimentary canal of the honeybee. If, as is usually assumed a specific enzyme is required for this process, then in some fashion the honeybee has elaborated this. In view of the peculiar distribution of this sugar in nature as at present known, this result is somewhat surprising.

The only insect other than the honeybee which might possibly use trehalose as a food is *Larinus nidificans* which produces the trehala manna and the larvae of various species of Diptera which live on various fungi. In view of the fact that trehalose, which is later used by it in the manufacture of manna, is found in such large proportions in the rectal content of the *Larinus* larva, one might venture the guess that this larva does not digest trehalose. There seems to be no available information concerning the ability of the various larvae found in fungi to digest the several carbohydrates found therein.

From the feeding results, it is suggested that bees contain the enzyme trehalase. The actual finding of an enzyme in an organism which has probably never encountered the substance acted upon until it fell into the hands of an experimenter, is not a new thing. Lactase has been found in the emulsin of almonds, or at least an enzyme has been found which hydrolyzes lactose, although it may act on the glucose element (1). Emulsin has been found in alimentary canals of vertebrates where it may produce toxic substances and where it is apparently not a useful enzyme. Bierry (12) (cited by Armstrong (1, p. 125)) found an enzyme which can hydrolyze  $\alpha$ -methyl galactoside in the digestive juices of *Helix*, although no compound of  $\alpha$ -galactose is known in nature. Kondo (43) "found that the liver is capable of synthesizing amino-acids from the corresponding ketonic acids and ammonia, even when the acids in question are not such as occur in the proteins of the organism. Since the one isomere is not of natural occurrence, but notwithstanding this it is attacked, it follows that, if a special enzyme is required, these organisms have produced in the course of their evolution an agent which has never had a chance of exerting its activity until the organisms fell into the hands of the physiologist" (6, p. 137). This quotation from Bayliss is here given because it seems probable that the hydrolysis of trehalose by the honeybee is another instance of the evolution of the ability to produce an enzyme, assumed to be

specific. It is probable, however, that never before in the evolution of the honeybee has trehalose been used as food, under which condition the enzyme could be utilized. Unless this is the case, one might assume that there is a source of trehalose as yet unknown which bees have at some time utilized as food; but from the character of the places where trehalose has so far been found, this seems rather improbable. However, since bees have been found collecting and utilizing melezitose (p. 16), one need not be surprised to find that they have used almost any carbohydrate at some time or another.

#### RAFFINOSE

In three feeding series (1, 2, and 4) the bees died about as soon on this sugar as did those without food. The difference is practically within the limits of one time the probable errors of the experiment. Bees are evidently not nourished by this sugar, although in both feedings they took considerable quantities, more than in the case of lactose, and even more per bee per day than in the case of some foods which maintained life for a time.

Raffinose belongs neither to the saccharose nor to the glucose groups of sugars but is a sacchoid ( $C_{18}H_{32}O_{16}$ ) or trisaccharide. By hydrolysis it is first split into levulose and melibiose, and by further hydrolysis the melibiose is split into glucose and galactose. The first splitting is presumably due to the action of invertase, the same enzyme which splits sucrose into levulose and dextrose, and which is so abundant in bees. If this action occurs in the case of raffinose fed to bees, it might be expected that the bees would be nourished by the released levulose. The second enzymic action is brought about by the specific enzyme melibiase, and as melibiose alone was not fed to bees there is nothing in these feedings to show whether the second enzyme is present. Melibiose divides into the same sugars as are produced by the action of lactase on lactose. One can at present draw no definite conclusions concerning the failure of raffinose to keep the bees alive.

#### MELEZITOSE

In the one feeding of melezitose (series 3) the bees lived well and were evidently able to utilize this sugar as food. While the death rate was slightly more rapid than in the case of bees fed commercial cane sugar (sucrose), the fact that one bee was still alive at the end of a month is definite proof that bees utilize the sugar.

On hydrolysis, melezitose is converted into turanose and glucose, and by further hydrolysis turanose yields glucose and levulose. Hudson and Sherwood (37) found that invertase is without any effect on a pure solution of melezitose. The enzyme which is capable of hydrolyzing this trisaccharide is not determined. By the use of acids the first step in hydrolysis is comparatively easy, but considerable difficulty is experienced in hydrolyzing turanose by means of acids. This is of interest in the present instance as it yet remains to be determined whether the bees simply hydrolyzed the melezitose to obtain glucose which nourished them, or whether they completed the process and hydrolyzed the turanose as well. The sluggish condition of the bees during the feeding suggests but does not do more than suggest, that they were accumulating turanose as feces. Turanose is hydrolyzed by an enzyme found in *Aspergillus niger* known as turanase.

## DEXTRIN

In the first feeding in which dextrin was given with cane sugar the average life of the bees was longer than in the case of bees without food. This mixture maintained life longer than commercial glucose, which contains relatively more dextrin than the food used, and was about the same as for brown sugar, which contains undetermined indigestible materials. In the second series, however, the cane sugar was omitted and the bees died sooner than did those without food, but within the probable error. The results in series 4 are the same as in series 2. Bees are evidently unable to maintain life on this carbohydrate. The dextrin used gave a slight blue color with a reddish tint when tested with iodine in potassium iodide. As there are doubtless many forms of dextrin in the transition of starch into maltose, it is impossible to say at just what stage of the hydrolyzing process in starch there is obtained a carbohydrate available to bees; but it is evident from the results of this feeding, as well as from other evidences, that most dextrans are not available to bees.

The inability of bees to utilize dextrin as food is a matter of primary importance in practical beekeeping. In the work done by Demuth and the author on wintering of bees, it was found that it was possible to cause the condition known as dysentery in wintering bees merely by giving honeydew or any dark honey and by confining the bees to the hive for long periods. The chief differences between the contents of dark honeys and the contents of lighter ones, on which dysentery is not contracted, are to be found in the dextrin content, although other materials are present in somewhat larger quantities in dark honeys than in lighter ones. In all this discussion an exception must be made of buckwheat honey which is quite satisfactory as a winter food for bees. The dark color in this honey presumably comes from tannin bodies, doubtless present in small quantities and not known to be injurious to bees. These facts suggested that dextrans in honeys are the actual cause of dysentery, and further suggested that in no case is any microorganism involved, as has been suggested. To determine the availability of dextrin, the first series of experiments here recorded was undertaken. Since in the first series dextrin was fed with sucrose, the result was inconclusive, and a second series was undertaken, in which, as has been seen, the bees died on dextrin as soon as if starved.

The literature on the digestion of bees, to which references have been made earlier, indicates that bees are able to digest starch, since the enzyme diastase has repeatedly been recorded as occurring in the alimentary canal of the bee. The term "diastase" is used in these cases presumably to include the whole series of enzymes which are capable of hydrolyzing starch into glucose, and dextrans are part of the series of compounds through which the starch passes in this series of changes. If therefore diastase is present in the bee one might expect that dextrin would serve bees as food. These matters will be treated at greater length under the discussion of the utilization of starch, in which it will be shown where the errors in former investigations have apparently been made.

Dextrans are the first soluble materials formed in the hydrolysis of starch in the plant, in which stage the carbohydrate material of the plant may circulate through plant tissues. This apparently

enables dextrin to pass through nectar glands and thus to enter the hive. If dextrin were actually hydrolyzed by bees, there might be reason to expect that in the honey-ripening process, this material would be changed into glucose. The variation in the dextrin content of honeys has already been mentioned. The presence of dextrans in ripened honeys and honeydew honeys is in itself circumstantial evidence of the inability of bees to provide the enzyme necessary for this transformation, at least in the honey stomach and salivary glands. As has been shown in the discussion of maltose, the enzyme which acts in the change of dextrin into maltose belongs to the amylase group, and apparently this entire group of enzymes is absent from the lumen of the alimentary tract of the bee. This will be discussed in detail under starch, in which discussion it is shown that amylase (or an allied enzyme) may be present elsewhere than in the lumen of the alimentary canal, and may thus help to explain the discrepancy between these results and those obtained by investigators who made enzyme extracts.

From these results the theory that dextrans are the cause of dysentery in wintering bees is greatly strengthened. Dysentery is so important in practical beekeeping that further investigations on this subject may well be carried on, especially since the work here recorded throws doubt on the presence of amylase or dextrases. It is certain, regardless of the final outcome of the work, that there is something in the darker grades of honey which is indigestible and that the accumulation of this material causes the condition so much dreaded by beekeepers. The theory of causation by a microorganism has so far nothing to support it. Of all the substances which increase in amount with the darkening of honeys, none is so evident as the increase in dextrans, which adds greatly to the validity of the theory that dextrans are the cause of dysentery. The various plant-coloring materials which occur to a greater extent in dark honeys, in association with dextrans, seem to be quite insufficient in quantity to account for the relatively enormous accumulations of feces in bees with dysentery; and that the quantity of feces to be accounted for is very large is well known to all beekeepers.

#### STARCH

This a complex polysaccharide, formed by the action of enzymes from many molecules of glucose, the starch molecule being exceedingly complex, and the number of constituent glucose molecules uncertain or variable. Starch is insoluble in water and in order that it be absorbed after digestion it must first be hydrolyzed by enzyme action (or by heat or acid artificially) until it reaches the stage of glucose again. That it may pass from place to place within a plant, it must be reduced at least to the stage of soluble dextrans. This reduction occurs in nature also through the actions of enzymes. As was stated under the discussion of dextrans, this cycle of enzyme action is presumed to depend on the successive action of several enzymes, all grouped together in the series of amylases (commonly called diastases). Starch grains are protected in plant tissues by a coating of cellulose, still more complex in molecular arrangement than starch itself. In order that the starch grain may be acted upon by amylase this coating must first be removed, either by the action of an enzyme, a cytase, or by other means. In the second series of

feedings, the coating was removed by cooking, while in the sixth series soluble starch which had been changed by some process that broke up the original starch grains into smaller units was used.<sup>12</sup>

In the first series, when potato starch was given with cane sugar, the average length of life was slightly greater (5.67 times that of the starved bees) than on any other food and the last bees died on the seventeenth day, at which time the last bees on honey also died. In the second series, potato starch was given in the form of a cooked paste and on this material the bees died as rapidly as if starved. The bees having dextrin died almost at the same rate as those on starch. This is interesting since, as observed in the discussion of dextrin, it is usually stated that bees have diastase in the alimentary canal. Petersen (59) states that he found diastase, but in his experiments he records nothing about the removal of the contents of the alimentary canal before the extraction of the enzymes; and because pollen is such a prolific source of diastase, the author was at first inclined to believe that the error in Petersen's work arose from the enzymes extracted from included pollen.

Since Petersen has stated positively that the bees contain within their alimentary canals the enzyme which serves to hydrolyze starch, it is desirable to examine his work in some detail. When he fed a solution of starch and sugar (heated for a time) which the bees accepted readily (see first series of experiments, p. 11), by means of iodine in potassium iodide, he traced the starch through the alimentary canal, found little of it in the rectal ampulla, and assumes that "a larger part" of it was converted into maltose or glucose. He was unable to demonstrate any change of starch into more soluble carbohydrates in the honey stomach, which is interesting in view of the claim of von Planta and others that there is diastase in honey. Not satisfied with the work previously done on this point Petersen performed the following experiment:

One gram of starch and 2 grams of NaF were dissolved in 100 c. c. of water with heat (solution I). Then 50 bee intestines were ground with 10 c. c. of equal parts glycerin and 1 per cent NaF solution, treated with toluol, and filtered. The filtrate 7 c. c. in quantity was brownish yellow and cloudy. He then made the following combinations:

- (1) 3 c. c. filtrate and 1 c. c. solution I.
- (2) 2 c. c. filtrate and 0.5 c. c. solution I.
- (3) 2 c. c. filtrate and 0.5 c. c. solution I.
- (4) Starch, solution I.

After 24 hours at room temperature No. 1 combination gave a red violet color with potassium iodide. After 24 hours more all four were tested with the following color results:

- (1) dark rose; (2) light rose; (3) dark rose; (4) blue.

No. 1 was then allowed to stand at a temperature of 40° C. and after 24 hours more gave a light red color with potassium iodide. As the result of these experiments Petersen states: "I consider the

<sup>12</sup> According to Jost (38, p. 149-150), the making of a paste of starch changes its chemical composition, as well as the physical appearance. "Employing the iodine test," he says, "we find that the original blue reaction rapidly gives place to a wine-red coloration. Finally, this latter reaction also disappears. Even without using iodine the fluid exhibits a marked alteration in appearance. Originally it is semi-fluid and opalescent; now it becomes transparent and quite watery. The starch, as such, has disappeared, and dextrin and maltose take its place. Maltose betrays its presence by the fluid being capable of reducing alkaline copper sulphate (Fehling's solution)."

breaking up of starch into dextrin as proven." He did not succeed in dissolving starch with the intestinal contents in a hanging drop.

The essential error in this experiment is that the author seemingly did not remove whatever pollen may have been in the intestines of the bees which he used in these experiments, and as most pollens contain diastatic enzymes, the whole experiment seems valueless.

After these experiments had progressed to series 4, the paper by Pavlovsky and Zarin (58) appeared, in which the statement was again made that the alimentary tract contains diastase. These investigators avoided the error which caused the present author to doubt the results obtained by Petersen, by removing the contents of the tract before extracting the enzyme. This necessitated still further work on starch, as a result of which the sixth series of feedings discussed in this paper was undertaken.

The feedings described for the sixth series were conducted, not to determine the death rate but to follow the course of ingested starch to ascertain whether any of it is modified by the digestive processes of the bee. To assist in the action of amylase, if present, these bees were not kept in a cool room but in ordinary room temperature<sup>13</sup>. They were not kept in a dark room but were exposed to a certain degree of stimulus from diffused, subdued light in order to induce somewhat greater activity and thus an increased need for food on the part of the bees. From time to time individual bees were removed while still living and the entire length of the alimentary canal was examined with Gram's iodine solution to determine whether any of the grains of soluble starch were in any way changed, as indicated by a change in color reaction with iodine.

If Pavlovsky and Zarin (58) are correct in stating that amylase is present in the lumen of the alimentary tract, then some of the grains of starch ingested as was the case in this series should stain red with iodine, others should take no stain with iodine, and still other grains should dissolve because the carbohydrate material has reached the stage of solubility. Whatever starch was actually rendered soluble would be invisible by the methods here used, but if diastatic action were actually occurring one should be able to find some starch grains taking a red stain with iodine and others taking little or no stain. If, on the other hand, every grain of starch stains deep blue with iodine, the conclusion seems obvious that the starch is not being acted upon by an enzyme.

Without going into the details concerning the examinations of individual bees, which were made several times a day as long as the bees lived, it may be said that up to the last day with the last bee, every ingested starch grain stained deep blue with iodine. It sometimes happened that the iodine would not at once penetrate the great mass of starch in the ventriculus and that the inner grains would therefore remain unstained or very slightly stained. Only the application of more iodine solution was necessary, however, to stain all the grains in the manner characteristic of unmodified starch.

<sup>13</sup> The temperature conditions prevailing in all these experiments were not especially favorable for the action of amylase. According to Jost (38) (figures after Kjelidahl (40, p. 121), at least for the amylase ferments found in plants, the action at 20° C. is about half that at 50° to 63° C. The maximum, minimum, and optimum points for different enzymes of the amylase group are not always the same. The temperature of the warmest part of a bee hive (consequently the approximate internal temperature of the bees in the hive) rarely exceeds 35° C. Bees away from the hive have a body temperature approximately the same as that of the surrounding air, varying between about 12° C. (at which temperature flight may begin) and 35° to 38° C.

This was true of the few grains of starch found in the honey stomachs of a few bees and of all grains in the ventriculus, small intestines, and rectal ampulla; and even the hindermost grains, probably those longest exposed to whatever enzyme action was present, stained the same as those just ingested. The conclusion is therefore unavoidable that this starch is not modified by an enzyme present in the lumen of the alimentary canal of the worker bee.

When reputable investigators announce the finding of diastase in the alimentary tract, and there can be no question of the accuracy of their statements; when one can trace starch through the entire alimentary tract and find none of it modified; and when bees having starch alone die as rapidly as if starved, there must of course be some explanation of the difference, and this explanation becomes unusually interesting. For several days during the progress of feeding in series 6, no suggested explanation was found. When the contents of the alimentary canal of one of the bees in series 6 was dissected the entire ventriculus was left on the slide and the iodine solution allowed to run about it. Some of the ventriculus lying in the microscopic field was found to be peculiarly stained. The muscles surrounding the ventriculus stained deep reddish brown, indicating the presence on these muscles of stored glycogen. While apparently there is still much work to be done on the enzyme reactions of glycogen, it is a common opinion that the enzyme splitting glycogen into glucose is the same enzyme which splits starch into glucose. Probably several enzymes are necessary in either case, but presumably also the entire series of enzymes are the same in both cases. It is further believed that an enzyme which can hydrolyze a carbohydrate also has the ability to cause that same carbohydrate to be built up from the component sugar molecules. In other words, enzymes are known to possess reversing action. On this assumption, the presence of stored glycogen in the muscles surrounding the ventriculus indicates the presence in these muscles of a diastase which enables the tissue to form glycogen. Obviously there could be no stored glycogen in these muscles without the action of the necessary enzyme.

Pavlovsky and Zarin (58) took the precaution to remove the contents of the alimentary canal, thus getting rid of the pollen therein contained, which is a prolific source of amylase. Herein they were on safer ground than Petersen (59) who says nothing about the removal of the contents. The Russian authors did not, however, remove the surrounding muscle layers from the alimentary canal, which would in fact be an impossibility, since the muscles are definitely incorporated in the tissues of the walls of the ventriculus. The presence of glycogenase in muscles is, of course, not at all surprising, certainly not in the bee, since it is well known that tissues of this insect store glycogen, notably the so-called fat body of both developmental and adult stages. As the situation now stands, therefore, there is no substantial evidence to support a belief that amylase actually occurs within the lumen of the alimentary canal of the honeybee. There is, on the other hand, rather definite circumstantial evidence that an enzyme does occur in a place which would invalidate all the experimental work so far recorded. Since, as has been stated at length, bees starve on starch alone and fail to modify a single starch grain in the alimentary canal, there seems reason to believe that under the conditions of the experiments

recorded, the lumen of the alimentary canal does not contain amylase and therefore has not the ability to hydrolyze starch or dextrin into glucose.

It still remains to explain the presence of amylase in honey, which can hardly be doubted in view of the considerable number of investigators who have reported finding it. Since amylase is a common plant enzyme and since the components of nectar are formed in the plant by the action of enzymes, of which amylase is one, there is the possibility that amylase is present in nectars and that it still remains after the ripening process. There seems to be nothing in the process of ripening honey which would tend to destroy or weaken amylase. Auzinger (2) definitely states that diastase is added from the salivary glands, but offers no proof of the statement. It is a rather safe assumption that he makes this statement on the basis of previous statements that diastase occurs in the alimentary tract of the bee. No certain explanation for the presence of amylase in honey seems possible at this time. There is reason, however, to question the statement that it has its origin in the bee itself. It may not be inappropriate to point out that honeys contain dextrans after the ripening process is completed. This indicates either that diastase is absent from honey or that hydrolysis has progressed until a balance has been reached. In that event one would assume that the original nectar had contained a considerable percentage of dextrans, for which there is no evidence. It may then be that the amylase extracted from honey is derived solely from included pollen grains, which it would be impossible to remove entirely by any known process.

The absence of amylase in the lumen of the alimentary canal is significant only because of the bee's inability to digest dextrans. Bees rarely get starch with normal food, and the only normal food which might contain starch is pollen. It is often stated that pollen contains no starch (59) but this is quite erroneous. There is considerable variation in the starch content of pollens, the same species having starch grains in some places and not in others. Some tropical plant species have starch grains in their pollen, but as a rule starch grains are present in the pollens of plants growing at high latitudes. Various grass pollens contain starch grains, and Sturtevant (72) has pointed out that the presence of these minute grains may cause confusion in the diagnosis of *Nosema* disease of adult bees.

The inability of bees to support life on starch is interesting further in view of the fact that in the spring of the year when brood rearing is heavy and when there is at times a shortage of this material beekeepers sometimes provide their bees with substitutes for pollen (56). Most of the materials used, such as corn, rye, and wheat flours, are rich in starch, which doubtless serves only for the formation of feces.

#### INULIN

In view of the failure of honeybees to utilize starch, it was thought desirable to test their ability to maintain life with another polysaccharide of about equal complexity which requires another enzyme for its hydrolysis. Inulin is hydrolyzed by inulase but it is also readily hydrolyzed by weak acids (21), only 0.05 to 0.2 per cent being required at a temperature of 40° C. The acidity of the ventriculus of the honeybee in terms of hydrogen-ion concentration is  $P_H$  6.8

to 7. Inulin belongs to the group of carbohydrates which on hydrolysis split into levulose (levulans), and the enzymes which act on them are collectively known as levulanases.

According to Straus (71), inulase is found in the feeding larvae of *Bombus mori* and *Hyponomota* sp., and is absent in five other species of Lepidoptera and Diptera studied. No inulase was found in the larvae of the species mentioned after they had ceased feeding, nor in the pupae or adults. Kobert (41) failed to find this enzyme in various invertebrates studied. For the higher animals the results obtained by various workers have apparently been in disagreement, but the more recent work has indicated that while inulase often fails to appear in the feces and while levulose does not appear in the urine of diabetic patients fed inulin, the disappearance of the inulin may be attributed to the fermentation of the inulin in the intestine without benefit to the organism (48, 53, 55). The literature on the hydrolysis of the levulasans has been well summarized by Swartz (73), from which it appears that with the exception of the two insects mentioned, no enzymes have been found in animals which split inulin.

It is therefore not surprising that the bees failed to be nourished by inulin fed them in the way employed in the present experiments. The bees which took this carbohydrate died as rapidly as did those without food. Pavlovsky and Zarin (58, p. 526), state that "the results obtained in our experiments allow us to conclude that the intestine of the bee does not produce inulase." In the table summarizing the results of their experiments they use a plus sign after inulase for the column headed "stomach," and a minus sign for all other parts of the alimentary tract. When the present author attempted to learn the cause of this discrepancy from the editor of the journal in which the paper by Pavlovsky and Zarin appeared he was informed that the use of the word "intestine" in the manuscript was not uniform. This word was sometimes used to designate the entire alimentary tract and sometimes to designate the intestine proper. The explanation of the discrepancy is therefore not clear.

#### GLYCERIN

Glycerin, a triose sugar, was tested in the series 4 feeding. The bees died as soon as if starved, in fact it was not certainly determined that they took any of the glycerin. While this material is sweet to the human taste the bees were apparently not attracted by it. It has several times been suggested that glycerin be used in the making of candy for shipping queen bees by mail, since the use of glycerin would prevent the drying of the candy. This single experiment suggests that it would be unsuitable for such a purpose; nor is there any reason to suspect its availability as food. The small quantity which would be used in such candy might do no harm, but it could hardly be desirable for the bees, since it would probably cause the formation of feces in the bees at a time when such material is highly detrimental.

#### RHAMNOSE

While it is hardly to be expected that bees will be able to utilize pentose sugars, it nevertheless seemed interesting to test these sugars. Rhamnose (a methyl pentose) therefore was used in feeding series 5. The bees died as rapidly as if starved, although they took some of the rhamnose offered them. This sugar does not occur free in nature but is a constituent of many glucosides and of flavone derivatives.

## XYLOSE

This is a pentose sugar, occurring commonly in a polysaccharide group, the pentosans, some of which have been found in honey. On this sugar (series 5) the bees died as rapidly as did those which had no food and no water. The length of life of bees fed on xylose was greater by seven times the sum of the two probable errors than that for the bees given no food but allowed water. This difference appears to have no significance.

## d-ARABINOSE

This is another pentose sugar, not fermented by yeast. Bees given this sugar in feeding (series 5) died almost as rapidly as those without food and as fast as did those without food and without water.

## l-ARABINOSE

Since l-Arabinose could also be obtained, it seemed interesting to determine whether the reverse molecular arrangement of this pentose sugar would have any different effect on the bees. They died when fed on this just as rapidly as did bees on the isomere.

## MANNITE

This carbohydrate alcohol has been found in manna, in the sap of the larch, and in other parts of plants, and also in honeydew (74). It seemed therefore of more than usual interest to determine whether bees may maintain life on it. The bees in series 5 which were given this carbohydrate lived 1.318 times as long as did those without food, or 8.9 times as long as the sum of the two probable errors. This is slightly longer than the other carbohydrates of this series, which did not benefit the bees, but is in sharp contrast with the results on sucrose and trehalose. The mannite doubtless failed to benefit the bees to which it was fed.

## AMYGDALIN

There was no reason to suspect that bees might be nourished by this glucoside, but it was tested to determine whether they have the ability to split it into its component parts. The material is bitter and readily soluble in water. When amygdalin is hydrolyzed by the action of the enzyme emulsin, an enzyme found in many species, it forms benzaldehyde, two molecules of glucose and hydrogen cyanide. It might be natural to assume, therefore, that if bees produce emulsin they would split this glucoside and thus release hydrogen cyanide, and that they would be poisoned thereby. If emulsin were present, it would be expected that the death rate from the splitting of amygdalin would be more rapid than from starvation. As a matter of fact, the death rate for bees fed amygdalin was practically the same as for the bees without food, which leads to the suspicion that emulsin is absent. Pavlovsky and Zarin (58) report the absence of emulsin with the use of amygdalin.

## SALICIN

Still another glucoside, salicin, was used in series 5 with the same result as was obtained with amygdalin. This is a natural glucoside, occurring in willow bark. It is hydrolyzed by emulsin to glucose and saligenin, which in turn yields salicylic acid on oxidation. This was tested by Pavlovsky and Zarin in their tests for emulsin, with negative results. In series 5 the bees died as rapidly as did those without food.

The testing of these two glucosides was of special interest in view of the fact that it has been reported that some poisonous honeys contain andromedotoxin, a glucoside found in plants of the Ericaceae. There are numerous vague reports of honeys from this group being poisonous to man, the supposition being that the contained glucosides are the poisonous element. It may first of all be stated that poisonous honeys are of exceedingly rare occurrence, so rare in fact as to be almost nonexistent. In one case of poisonous honey from a Southern State, the evidence pointed to one of the Ericaceae as the source of the nectar. It was impossible, however, to identify pollen grains of that species in the honey. Neither was it possible chemically to detect the presence of the glucoside in the honey. In spite of these facts the honey caused distressing symptoms in those who ate it. Fortunately it had a bitter taste so that no one ate enough of it to be harmed seriously. The bees were observed working freely on this species, but there was not the slightest evidence that they were injured by the material gathered, as might have been the case if they had been able to hydrolyze the glucoside andromedotoxin, which contains hydrogen cyanide as a constituent.

#### COMMERCIAL GLUCOSE

This substance, sometimes called corn sirup, is a mixture of dextrin, glucose, and maltose, formed by the action of acids on starch. The same action may occur through the action of amylase. As will be seen from the data on glucose and maltose, either or both of these sugars are capable of nourishing bees, but as will be seen from the discussion of dextrin, this is not utilized for bees, forms feces in abundance, and causes activity on the part of the bees which wears them out and causes premature death. In the first feeding of commercial glucose the bees lived 2.48 times as long as did the starved bees. In the second feeding they died about as soon as the bees without food, but lived a little longer than did the bees on commercial glucose in the first feeding. What nourishment the bees received from this substance was doubtless from the glucose and maltose.

When the manufacture of commercial glucose was first begun on an extensive scale in the United States many beekeepers tried feeding it to their bees in winter. In all recorded cases dysentery developed and the losses of colonies on this food was heavy. The first difficulty encountered was that bees did not take it readily, either because of its lack of sweetness or of the presence of some taste disagreeable to them. Bees will often starve with commercial glucose available to them. Especially in the first feeding, the amount of commercial glucose taken by the bees was smaller than for other foods on which they lived. In the second feeding, when the amounts of all the foods taken were smaller, the bees on commercial glucose died almost as rapidly as did those without food.

#### BROWN SUGAR

In the first feeding the bees lived considerably longer on this food than did those without food, while in the second feeding they died almost as soon as did the bees without food. Bees are obviously able to utilize sucrose which is the chief constituent of this sugar; and the fact that they died sooner on this than on chemically pure

sucrose indicates that the other constituents are less suitable. The brown sugar used was not analyzed. It was medium brown. Brown sugars contain in addition to sucrose, some reducing sugars (3 to 6 per cent), considerable ash (0.8 to 3 per cent), and moisture. There are also present certain dextrins, probably the constituents which are undesirable from the standpoint of bee feeding.

That brown sugar is totally unsatisfactory as food for bees in winter confinement was clearly shown during the period of the war when sugar was scarce. An extensive beekeeper in one of the Northern States unable to get granulated sugar, fed some light brown sugar with the result that he lost most of the colonies so fed. Since this beekeeper is highly skilled in his work, there is no reason to suspect any other cause of the death. Bees not so fed came through the winter in fine condition.

#### HONEYDEW HONEY

In the first feeding the bees lived almost as long on this food as they did on cane sugar or honey, and they are therefore nourished by it. The amount of dextrins present was not sufficient to cause much earlier death under the conditions of the experiment. In the second feeding the bees died almost as soon as those without food. Whether this sample of honeydew honey contained melezitose in addition to glucose, levulose, and sucrose does not appear to be essential, as bees are able to utilize all these sugars. Any detrimental effect resulting from this food doubtless came from the dextrins. The effect of honeydew honey on bees confined for a longer period in winter has been described (63).

#### HONEY

As is to be expected, the bees on this food lived as long as those on any food. In the second series the bees on levulose lived slightly longer, but the difference is well within the range of the probable errors. In the first feeding the bees on starch and cane sugar mixture lived longer than those on honey, but almost within once the probable errors. Because of the dextrin content, the honey used in these feedings would be considered of rather inferior quality for market as well as for use by bees in winter, but for the most part the bees made a good showing on it.

#### CONCLUSIONS

The sources of food as well as the feeding habits of the honeybee are specialized. Because of the total dissimilarity between the structure and physiology of the alimentary canal of the bee and of higher animals, analogies of function between the parts of the alimentary canal, such as the application of identical names to supposedly homologous parts, are exceedingly unwise. The foregoing experiments also show that physiological analogies between bees and higher animals are as unwarranted as are anatomical ones. Even in closely related insects, where there are closely homologous structures in the alimentary canal, it is unwise to assume the same abilities to absorb and assimilate certain materials as food, because of homologous structure and apparent physiological homologies. Because of the great diversity of foods used, enzymes of all kinds may be assumed to exist among insects. In some instances the enzymes have been

found, but this is no basis for assuming that they will also occur in related species. Because of the diversity of feeding habit, it seems necessary to determine the availability of various materials for almost every species separately and to avoid guesses from analogy.

In the present studies, only the carbohydrates have been investigated for their availability as food for bees. This is possible by the method used, because bees frequently remain alive and active for a considerable time with no food other than carbohydrates.

Of the monohexose sugars, bees utilize as food glucose and levulose, but not galactose and probably not mannose. Of the disaccharides they utilize sucrose, trehalose, and maltose but not lactose. Of the trisaccharides they utilize melezitose but not raffinose. They fail to utilize as food the more complex polysaccharides dextrin, starch, or inulin. It is evident from the fact that they do not die more rapidly when given the glucosides containing hydrogen cyanide as a constituent that bees lack the enzyme emulsin. They do not utilize glycerin, mannite, or the four pentose sugars, rhamnose, xylose, *d*-Arabinose, and *l*-Arabinose. They can live on commercial glucose for a time from the dextrose and maltose present, but show a pronounced dislike for this material, and it does not serve as a safe food. They can live on brown sugar, but practical experience in the apiary shows its unsuitability for winter feed. Honeydew contains sugar which bees utilize but it is unsafe for winter. Honey, even the rather inferior type used in this experiment, is as good as any food material for bees, and there is reason to believe, from data not herein contained, that it is far better for bees at certain times of year than any artificial sugar obtainable.

Interpreting these findings in terms of enzymes, it appears that the honeybee contains invertase, maltase, and trehalase but not lactase, emulsin, inulose, amylase (diastase), and dextrase, if this is to be separated from amylase. No evidence is herein contained on enzymes other than those which are concerned in the hydrolizing of carbohydrates.

From the point of view of practical beekeeping, the absence of enzymes of the amylase-dextrase group is of first importance, and the work here recorded supports the theory that dysentery in winter is caused by the indigestibility of dextrans in the winter food supply, and the consequent accumulation of this material in the rectal ampulla as fecal material.

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# THE UTILIZATION OF CARBOHYDRATES AS FOOD BY HONEYBEE LARVAE<sup>1</sup>

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## INTRODUCTION

The food of honeybee larvae, as of animals in general, consists of mixtures of carbohydrates, proteins, fats, mineral salts, water, and presumably vitamins. But, of course, this statement does not mean that the larvae can utilize all the carbohydrates, all the proteins, all the fats, etc. Thus far, however, no one has reported any attempt to discover which particular ones from each of these classes of foods the larvae can use.

Since carbohydrates constitute one of the most important classes of foods, and since they are for the most part well known chemically, it has seemed to the writer logical to begin the consideration of this problem with an investigation of the ability of the larvae to use carbohydrates as food.

This investigation seemed worth while for two reasons: (1) It might be of practical value to the beekeeper to know which carbohydrates the larvae can utilize, since during the latter part of their larval life workers and drones are fed large quantities of carbohydrates, often furnished by the beekeeper; (2) from the point of view of comparative physiology it would be of interest to know how honeybee larvae compare with honeybee adults, and also with other insect larvae and adults, in their ability to utilize certain carbohydrates.

## REVIEW OF PREVIOUS WORK ON DIGESTION OF CARBOHYDRATES IN INSECTS

A review of the literature regarding carbohydrate digestion in insects reveals, as mentioned above, no previous work on honeybee larvae, but shows considerable work on honeybee adults and on larvae and adults of other insects in several of the orders. (Table 1.) Most of the work has been a search for enzymes in the various portions of the alimentary tract. Usually an extract was made of the organ under investigation and the action of this extract was tested on various carbohydrates. According to this method, if a carbohydrate was hydrolyzed by the extract, the enzyme peculiar to that process was supposed to be present.

In a few instances, in the investigations of Cleveland (?),<sup>4</sup> Sieber and Metalnikow (21), Chapman (6), and Phillips (17), a direct feeding

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<sup>2</sup> The writer thanks E. F. Phillips, formerly senior apiculturist; the present members of the staff of the bee culture laboratory; and S. O. Mast, of Johns Hopkins University, for valuable suggestions and assistance during the course of the investigations herein reported.

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<sup>4</sup> Reference is made by number (italic) to "Literature cited," p. 451.

method was employed, a group of insects being fed on a certain carbohydrate until all of its members died and the length of life of such a group being compared to that of other groups kept as checks. If the group fed on a certain carbohydrate lived longer than a similar, starved group, its members were supposed to possess the ability to utilize that carbohydrate as food.

TABLE 1.—A summary of investigations on the digestion of carbohydrates by insects

Name of insect	Part investigated *	Investigator	Date	Results of tests made for the following enzymes:							
				Amylase	Invertase	Maltase	Lactase	Cellulase	Inulase	Raffinase	Emulsin Others
Orthoptera:											
Blatta orientalis.	Extract of salivary gland.	Basch (2)	1858	+							
Do.....	Extract of walls of mid-gut.	do	1858	+							
Do.....	Secretion of salivary glands.	J. de Bellesme (11).	1876	+							
Do.....	Secretion of gastric coeca.	do	1876	—							
Do.....	Secretion of Malpighian tubes.	do	1876	—							
Do.....	Extract of salivary glands.	Swingle (24).	1925	+	—	—	—				
Do.....	Extract of fore-gut.	do	1925	—	+	+	—				
Do.....	Extract of gastric coeca.	do	1925	—	+	+	—				
Do.....	Extract of mid-gut.	do	1925	—	+	+	—				
Do.....	Extract of hind-gut.	do	1925	—	—	—	—				
Periplaneta americana.	Pulverized salivary glands.	Plateau (19).	1876	+							
Stethophyma grossum.	Extract of salivary glands.	Plateau (18).	1874	+							
Locusta viridissima.	do	do	1874	+							
Do.....	Extract of salivary glands, pupa.	do	1874	+							
Do.....	Extract of salivary glands, larva.	do	1874	+							
Isoptera:											
Reticulitermes flavipes.	Ability to live	Cleveland (7).	1924					—			
Hemiptera:											
Cicada sp.	Extract of whole gut.	Axenfeld (1).	1904	—	+						
Aphrophora salicis (De Geer).	Extract of salivary glands, larva.	Gruner (10).	1900	+							
Do.....	Extract of alimentary tract, larva.	do	1900	—							
Nepa cinerea.	Extract of salivary glands.	Plateau (18).	1874	+							
Ranatra linearis.	do	do	1874	+							
Notonectes sp.	Extract of whole gut.	Axenfeld (1).	1904	—	+						
Lepidoptera:											
Liparis dispar.	Secretion of mid-gut of larva.	Plateau (18).	1874	+							
Pieris brassicae.	Extract of gut of larva.	Biedermann (4).	1910	+							
Do.....	Extract of gut, adult.	Axenfeld (1).	1904		+						
Tineola bisselliella.	Excrement of larva.	Sitowski (22).	1905	(b)				—			
Galleria mellonella.	Ability to live.	Sieber and Metainikow (21).	1904	—	—						
Do.....	Extract of intestine of larva.	do	1904	+							
Do.....	Extract of whole feeding larva.	Straus (23).	1909	+	+				—	+	

\* The adult insect is referred to unless otherwise stated.

b Very little.

TABLE 1.—A summary of investigations on the digestion of carbohydrates by insects—Continued

Name of insect	Part investigated	Investigator	Date	Results of tests made for the following enzymes:							
				Amylase	Invertase	Maltase	Lactase	Cellulase	Inulinase	Raffinase	Emulsin Others
Lepidoptera—Continued.											
Euproctis chrysorrhoea.	Extract of whole feeding larva.	Straus (23)	1909	+	+	+	+	?	+		
Do.	Extract of whole pupa.	do.	1909	+	+		?		+		
Oenieria dispar.	Extract of whole feeding larva.	do.	1909	+	+	+					(1)
Bombyx neustria.	do.	do.	1909	+	+				+		
Do.	Extract of whole pupa.	do.	1909	+	+	+			+		
Bombyx mori.	Analysis of food and excrement.	Kellner (12)	1884								
Do.	Extract of whole feeding larva.	Straus (23)	1909	+	+		+		+	+	
Do.	Extract of whole not-feeding larva.	do.	1909	(e)	+						
Do.	Extract of intestine of larva.	Axenfeld (1)	1904								+
Do.	Extract of whole pupa, 10 days.	Straus (23)	1909	+	+	+				+	
Do.	Extract of whole pupa, 14 days.	do.	1909	+	+	(e)				+	
Do.	Extract of whole pupa, 18 days.	do.	1909		+					+	
Do.	Extract of whole pupa, 21 days.	do.	1909	+							
Do.	Extract of whole adult.	do.	1909	{ + }	+						
Hypnomena sp.	Extract of whole feeding larva.	do.	1909	+	+	+	+		+	+	
Do.	Extract of whole pupa.	do.	1909	+	+	?			+	+	
Carpocapsa pomonella.	Extract of gut of larva.	Axenfeld (1)	1904		+						
Vanessa sp.	Extract of gut.	do.	1904		+						
Vanessa urticae.	Extract of whole pupa.	Straus (23)	1909			+					
Diptera:											
Calliphora vomitoria.	Extract of whole feeding larva.	do.	1909	+	+	+				+	
Do.	Extract of whole not-feeding larva.	do.	1909	+	+	?				+	
Do.	Extract of whole pupa.	do.	1909	+	+	+				+	
Do.	Extract of whole adult.	do.	1909	+	+	+				+	
Do.	Extract of excrement.	Weinland (26).	1906								
Do.	Extract of whole adult.	Weinland (26).	1907	+							
(Musca) Sarcophaga carnaria.	Extract of gut of larva.	Axenfeld (1)	1904								
Do.	Extract of gut of adult.	do.	1904		+						
Musca domestica.	Extract of whole adult.	Kobert (15)	1903	+							
Coleoptera:											
May beetles.	do.	do.	1903	+							
Tenebrio molitor.	Contents of mid-gut of larva.	Frenzel (9)	1882	+							
Do.	Extract of mid-gut of larva.	Biedermann (5).	1898	+	+	+					
Carabus sp.	Extract of whole gut.	Axenfeld (1)	1904		(1)						
Dytiscus dimidiatus.	Fluid in crop of adult.	Plateau (18)	1874								
Do.	Extract of mid-gut.	do.	1874								
Do.	Extract of small intestines.	do.	1874								

\* Both in gut and in body.

\* In the gut; questionably, in the body.

\* Question marks in these columns mean doubtfully positive results.

/ No agar ferment.

\* Little.

\* For glycogen diastase.

\* For starch diastase.

\* Weak.

TABLE 1.—A summary of investigations on the digestion of carbohydrates by insects—Continued

Name of insect	Part investigated	Investigator	Date	Results of tests made for the following enzymes:							
				Amylase	Invertase	Maltase	Lactase	Cellulase	Inulase	Raffinase	Emulsin
Coleoptera—Con.											
Dytiscus sp.	Extract of whole gut	Axenfeld (1)	1904	—	(1)	—	—	—	—	—	—
Dytiscus marginalis.	Fluid in crop of adult	Plateau (18)	1874	—	—	—	—	—	—	—	—
Phymatodes variabilis.	Extract of mid-gut of larva.	Seillière (20)	1905	—	—	—	—	—	—	—	(4)
Tribolium confusum.	Ability to live	Chapman (6).	1924	+	—	—	—	—	—	—	—
Oryctes nasicornis.	Extract of mid-gut of adult.	Plateau (18)	1874	+	—	—	—	—	—	—	—
Melolontha vulgaris.	do.	do.	1874	+	—	—	—	—	—	—	—
Melolontha sp.	Extract of intestine	Axenfeld (1)	1904	—	(1)	—	—	—	—	—	—
Hydrous caraboides.	Fluid in oesophagus	Plateau (18)	1874	+	—	—	—	—	—	—	—
Do.	Contents of mid-gut	do.	1874	+	—	—	—	—	—	—	—
Do.	Extract of mid-gut wall.	do.	1874	+	—	—	—	—	—	—	—
Hydrophilus piceus.	Fluid in oesophagus	do.	1874	+	—	—	—	—	—	—	—
Do.	Contents of mid-gut	do.	1874	+	—	—	—	—	—	—	—
Do.	Extract of mid-gut wall	do.	1874	+	—	—	—	—	—	—	—
Hydrophilus sp.	Extract of intestine	Axenfeld (1)	1904	—	+	—	—	—	—	—	—
Hymenoptera:											
Ants.	Extract of whole pupa	Kohert (13)	1903	+	—	—	—	—	—	—	—
Apis mellifica	Extract of head	Erlenmeyer (8).	1874	+	+	—	—	—	—	—	(1)
Do.	Extract of thorax	do.	1874	(1)	(1)	—	—	—	—	—	—
Do.	Extract of abdomen	do.	1874	+	+	—	—	—	—	—	—
Do.	Extract of fore-gut	Axenfeld (1)	1904	—	+	—	—	—	—	—	—
Do.	Extract of mid-gut and of hind-gut.	do.	1904	—	(1)	—	—	—	—	—	—
Do.	Known substance fed; analyzed in mid-gut.	Petersen (16)	1912	(1)	+	—	—	—	—	—	—
Do.	Known substance fed; analyzed in fore-gut.	do.	1912	—	+	—	—	—	—	—	—
Do.	Extract of mid-gut	do.	1912	+	—	—	—	—	—	—	—
Do.	Extract of fore-gut	Pavlovsky and Zarin (15).	1922	—	—	—	—	—	—	—	—
Do.	Extract of ventriculus	do.	1922	+	+	—	—	—	—	—	—
Do.	Extract of small intestine	do.	1922	—	—	—	—	—	—	—	—
Do.	Extract of large intestine	do.	1922	—	—	—	—	—	—	—	—
Do.	Ability to live	Phillips (17)	1924	—	+	+	—	—	—	—	(m)

1 Weak.

2 Xylanase.

3 Zymase.

4 Melenzitate; trehalase.

1 Weak.

2 Xylanase.

3 Zymase.

m Melezitase; trehalase.

For the purpose of comparison, the results of the various investigators are brought together and presented in Table 1. This table gives the name of the insect, the part of the insect investigated, the method, the name of the investigator, the year, and a list of all the carbohydrate-digesting enzymes found. The insects are listed under their respective orders, but no attempt is made to classify the insects within an order.

Regarding the use and scope of Table 1 attention should be called to the following facts:

1. The table merely indicates in any case that the enzyme was, or was not, found, and does not usually indicate its quantity or intensity.

2. Negative results mean simply that the investigator tested for the enzyme in question and did not find it. It may be that the enzyme was present in quantities too small to be detected by the test used.

3. In the case of feeding experiments it is assumed that the ability to use a substance as food means that the enzyme necessary to digest that food is present.

4. A host of insects whose feeding habits are well known in a general way, but whose digestive abilities have not been specifically investigated, are for obvious reasons excluded from the table.

One may briefly summarize as follows the results of previous investigations regarding the ability of insects in general to digest carbohydrates:

1. Amylase is very widely distributed among insects, having been demonstrated in nearly every species investigated, but is often localized in a particular organ.

2. In but very few species has the insect been found entirely lacking in any of the common carbohydrate-digesting enzymes, i. e., amylase, invertase, and maltase.

3. No insect investigated has been shown to be able to digest cellulose. It is quite probable, however, that many insects not investigated can do so.

4. In the case of most species every enzyme present is of direct use in digesting the food which the insect eats.

5. With some few species, however, enzymes occur which are probably never used, e. g., amylase in ant pupae and trehalase in honeybees.

6. In the case of a few other species substances are habitually eaten for which the insect secretes no digesting enzymes, e. g., wood by termites.

In regard to honeybees in particular, the review of the literature shows the following facts (see Table 1, *Apis mellifica*):

1. All work of this sort has been done on adults; none on the larvae.

2. All work except Phillips's and part of Petersen's has been done by the method of extracts.

3. All investigators using the method of extracts have found amylase somewhere in the animal, yet Phillips found no ability to utilize starch as food.

4. All investigations have revealed invertase somewhere in the animal.

5. In two investigations lactase, inulase, and emulsin were sought, either directly or indirectly, but were not found.

6. From one investigation it appears that cellulase is not present.

7. From one investigation it appears that bees can readily utilize the rare sugars, melezitose and trehalose.

The problem now is to ascertain how the larvae of honeybees compare with adult honeybees and with other insects in their ability to utilize carbohydrates as food.

#### MATERIAL AND METHOD

Two methods of investigation were open in approaching this problem, the method of making extracts of various organs and testing the action of these extracts on various carbohydrates, and the method of feeding a group of larvae on a given carbohydrate and ascertaining whether or not the larvae receive any nourishment from it, by comparing the length of life of this group with that of a similar group fed on water only.

The latter method, the direct method, was chosen for two reasons: (1) It enabled one to test any number of carbohydrates in a direct manner, without encountering the technical errors present in many of the chemical tests for carbohydrates; in fact, there are as yet no specific chemical tests for many carbohydrates; (2) the results discovered by direct feeding would seem to be of more practical value than results obtained by the method of extracts, since in the former only those enzymes are considered which are of actual use in keeping the larva alive.

In performing an experiment to compare the length of life on a given food with the length of life on water alone the procedure was as follows: Enough larvae of as uniform size as possible were removed from the comb to serve for the whole experiment. As each larva was taken from the comb with a transferring needle it was floated out on water, where any food clinging to it was washed off. After a sufficient number of larvae had been removed they were divided into lots of 10 to 25 each, and each lot was weighed. The weight was usually about 18 mgm. per larva, although in some experiments the average was as low as 11 mgm. and in others as high as 46 mgm. According to Nelson and Sturtevant (14), the weight of larvae at hatching averages 0.100 mgm.; at 1 day of age, 0.650 mgm.; at 2 days, 4.745 mgm.; at 3 days, 24.626 mgm.; and at 4 days, 93.990 mgm.; it follows that larvae weighing 18 mgm. are, on an average, nearly 3 days old.

After weighing, the larvae were transferred to a small watch glass containing a solution of the carbohydrate to be tested; the watch glass was placed inside a Petri dish containing a little water, to serve as a moist chamber; and the whole was put inside an incubator at about hive temperature (35° C.). An experiment consisted of a number of such lots of similar larvae, fed on various concentrations of the food in question, together with one lot supplied with water only, as a check. For most of the lots examinations were made at intervals of six hours, the dead being removed at this time and the food replaced by a fresh supply if it appeared in the least cloudy. A larva was considered dead if it no longer responded by muscular contraction when touched with the transferring needle. The time at which death occurred was assumed to have been midway between the time of the examination at which it was found dead and the time of the last previous examination. The carbohydrates used were dextrose, levulose, galactose, sucrose, maltose, lactose, trehalose, melezitose, starch, glycogen, and dextrin, in various concentrations, and, finally, a series of honeys. Lots were run in groups of 5 to 10 at a time, and a starvation check, in which distilled water alone was fed, was run with each group. The expressions "starvation check" and "water check" are used interchangeably throughout this paper. Often two or three such groups or series were run for a single carbohydrate.

The complete record of a typical lot, as given in Table 2, will make clear the procedure in each case.

Other lots fed at the same time and under similar conditions had, as average lengths of life, those fed on 5 per cent sucrose,  $11.80 \pm 0.62$  hours; 15 per cent sucrose,  $17.98 \pm 1.18$  hours; 25 per cent sucrose,  $32.90 \pm 2.95$  hours; 50 per cent sucrose,  $59.66 \pm 4.23$  hours; and water,  $8.68 \pm 0.15$  hours.

TABLE 2.—A complete record of all the observations and computations made for a typical lot of honeybee larvae <sup>a</sup>[Lot S3; food, 35 per cent sucrose; <sup>b</sup> weight, 0.0185 gm. each]

Date, 1924	Hour	Number of larvae alive	Number of larvae died since last examination (=n)	Length of life (hours) (=l)	n×l
Aug. 18.	12 m.	20			
Do.	6 p. m.	19	1	3	3
Do.	11 30 p. m.	19			
Aug. 19.	6 a. m.	19			
Do.	12 m.	18	1	21	21
Do.	6 p. m.	18			
Do.	11.30 p. m.	18			
Aug. 20.	6 a. m.	18			
Do.	12 m.	15	3	45	135
Do.	6 p. m.	11	4	51	204
Do.	11.30 p. m.	6	5	57	285
Aug. 21.	6 a. m.	4	2	63	126
Do.	12 m.	2	2	69	138
Do.	6 p. m.	2			
Do.	12 p. m.	1	1	81	81
Aug. 22.	6 a. m.	0	1	87	87
Total.			20		1,080

<sup>a</sup> All other lots in these experiments were treated in a similar way.<sup>b</sup> Thirty-five per cent sucrose means 35 gm. of pure sucrose crystals in 100 c. c. of solution. All solutions mentioned were made in the same way.

Mean length of life, 54 hours.

Probable error of the mean (Peters' formula), 2.39 hours.

It was possible in this way to get a comparison of the effect of any concentration of any carbohydrate desired on the length of life of the larvae.

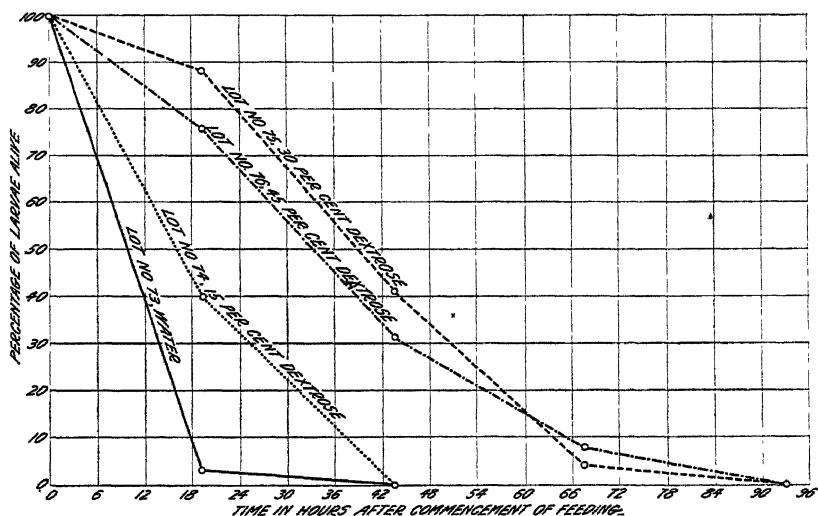


FIG. 1.—Percentages of survivors of lots of honeybee larvae, at intervals after the commencement of feeding, fed on solutions of dextrose in certain concentrations with water, and of one lot supplied with water only

The data obtained in these feeding experiments are presented in Tables 3 to 22 and Figures 1 to 11. The experimental results are

given under the headings monosaccharides, disaccharides, trisaccharides, polysaccharides, and honeys. Following these a section

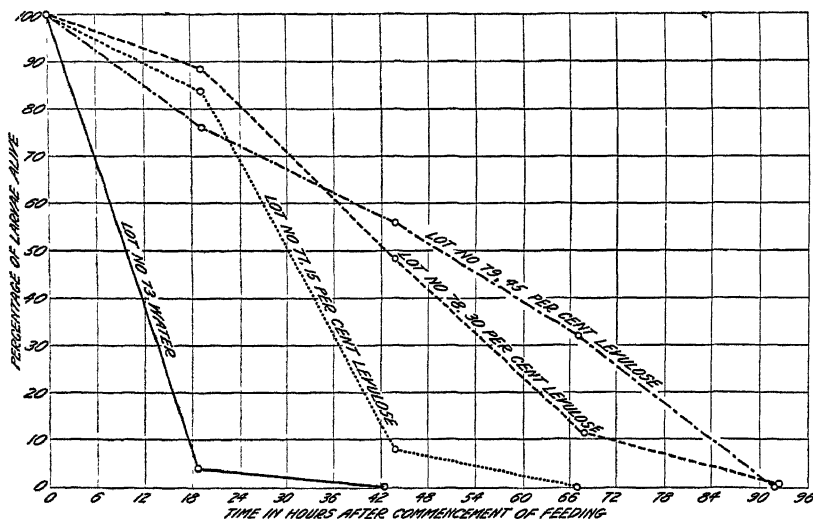


FIG. 2.—Percentages of survivors of lots of honeybee larvae, at intervals after the commencement of feeding, fed on solutions of levulose in certain concentrations with water, and of one lot supplied with water only

is devoted to a review and a comparison of those lots, showing optimum length of life for each carbohydrate used.

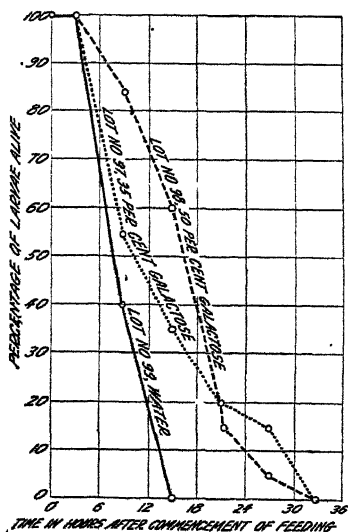


FIG. 3.—Percentages of survivors of lots of honeybee larvae, at intervals after the commencement of feeding, fed on solutions of galactose in certain concentrations with water, and of one lot supplied with water only

## EXPERIMENTAL RESULTS

### MONOSACCHARIDES

Of the monosaccharides, the simplest of the sugars, only the three common representatives were tested—dextrose, levulose, and galactose. All lots fed on dextrose or levulose outlived the larvae fed on water, lot 75, in 30 per cent dextrose, living about four times as long, and lot 79, in 45 per cent levulose, about four and a half times as long, as the lot fed on water (Tables 3, 4, and 5, and figs. 1 and 2). Most of the lots fed on galactose likewise outlived the water check, lot 98, on 50 per cent galactose, living nearly twice as long as lot 99 on water. (Tables 6 and 7 and fig. 3).

It seems evident, then, that the larvae can get nourishment from these simple sugars. The next question is, can the larvae digest other, more complex carbohydrates, changing them to these simple forms? If they sustain themselves on these other carbohydrates it

will be considered evidence that they do digest them; if they die in as short a time as they die when starved, it will appear that they do not digest these carbohydrates.

TABLE 3.—Observations to ascertain the relative duration of life of representative lots of honeybee larvae fed on dextrose in specified concentrations with water, and a lot supplied with water only, as a check <sup>a</sup>

Lot 73 (water)		Lot 74 (15 per cent)		Lot 75 (30 per cent)		Lot 76 (45 per cent)	
Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving
Hours	Per cent	Hours	Per cent	Hours	Per cent	Hours	Per cent
18.5	4	19.5	40	19.5	88	19.5	76
42.5	0	43.5	0	44.5	40	44.0	32
				67.5	4	67.0	8
				93.0	0	92.5	0

<sup>a</sup> Data presented graphically in fig. 1.TABLE 4.—Observations to ascertain the relative duration of life of representative lots of honeybee larvae fed on levulose in specified concentrations with water, and a lot supplied with water only, as a check <sup>a</sup>

Lot 73 (water)		Lot 77 (15 per cent)		Lot 78 (30 per cent)		Lot 79 (45 per cent)	
Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving
Hours	Per cent	Hours	Per cent	Hours	Per cent	Hours	Per cent
18.5	4	20.0	84	20.0	88	20.0	76
42.5	0	44.5	8	44.5	48	44.0	56
		67.0	0	67.5	12	67.0	32
				92.5	0	92.0	0

<sup>a</sup> Data presented graphically in fig. 2.

TABLE 5.—Average length of life for lots of larvae fed respectively on solutions of dextrose and of levulose and on water

Lot No.	Food	Number of larvae	Weight of each	Length of life
			Mgm.	Hours
73	Water.....	25	19	10.10±0.55
74	15 per cent dextrose.....	25	18	18.27±1.43
75	30 per cent dextrose.....	25	16	39.90±2.32
76	45 per cent dextrose.....	25	18	36.01±2.73
77	15 per cent levulose.....	25	19	30.57±1.58
78	30 per cent levulose.....	25	18	39.06±2.12
79	45 per cent levulose.....	25	19	47.56±3.64

TABLE 6.—Observations to ascertain the relative duration of life of representative lots of honeybee larvae fed on galactose in specified concentrations with water, and a lot supplied with water only, as a check <sup>a</sup>

Lot 97 (35 per cent)		Lot 98 (50 per cent)		Lot 99 (water)	
Time	Surviving	Time	Surviving	Time	Surviving
Hours	Per cent	Hours	Per cent	Hours	Per cent
3.0	100	3.0	100	3.0	100
9.0	55	9.0	85	9.0	40
15.0	35	15.0	60	15.0	0
21.5	20	21.5	15		
27.0	15	27.0	5		
32.5	0	32.5	0		

<sup>a</sup> Data presented graphically in fig. 3.

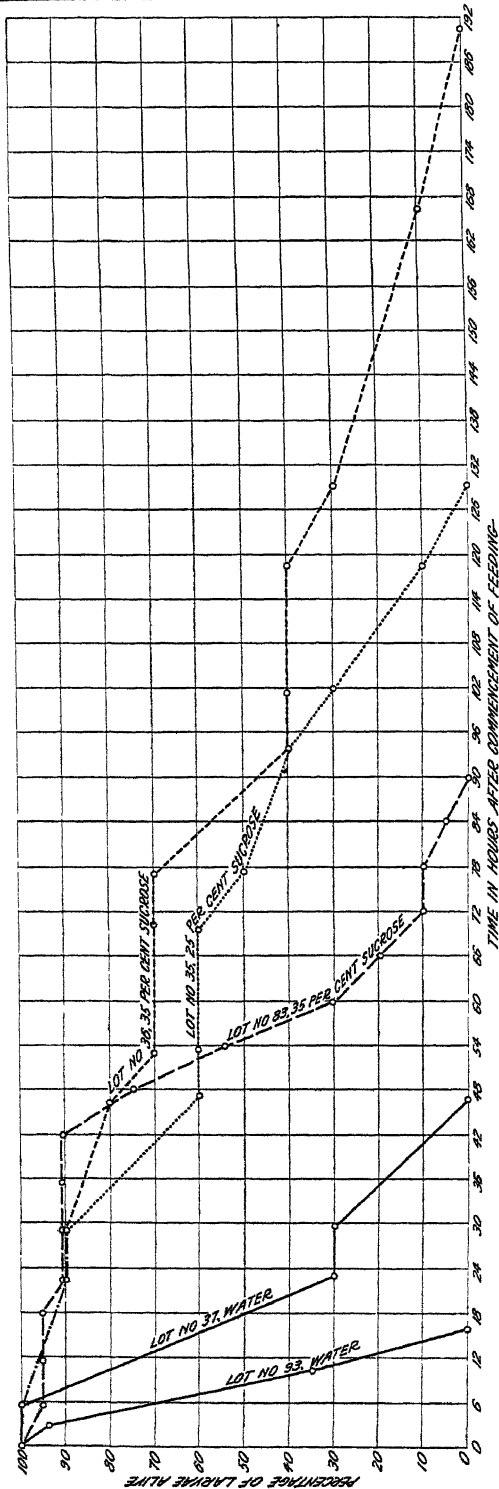


FIG. 4.—Percentages of survivors of lots of honeybee larvae, at intervals after the commencement of feeding, fed on solutions of sucrose in certain concentrations with water, and of one lot supplied with water only

# DISACCHARIDES

The disaccharides commonly used as food by animals are sucrose, maltose, and lactose. Honeybee larvae were fed on all of these, and also on trehalose, to which particular interest is attached in connection with the honeybee. This rare sugar occurs, so far as known, only in a few fungi. The interest here lies in the fact that Phillips found adult honeybees to be able readily to utilize this sugar as food, in spite of the fact that it probably never occurs in their normal diet.

In the experiments here described the larvae lived longer on all concentrations of sucrose than on water, the concentration of 75 per cent being the most favorable; the ratio of the length of life of lot 125 (75 per cent sucrose) to lot 129 (water) is about 15 to 1, that of lot 84 (50 per cent sucrose) to lot 93 (water) 7 to 1, and that of lot 36 (35 per cent sucrose) to lot 37 (water)  $4\frac{1}{2}$  to 1. (Tables 8 and 9 and fig. 4.)

On maltose the larvae likewise outlived decidedly the larvae that were starved; e. g., lot 41, on 35 per cent maltose, lived more than five times as long as lot 43, on water, and lot 88, on 35 per cent maltose, lived more than three times as long as its water check, lot 93. (Tables 10 and 11 and fig. 5.)

TABLE 7.—Average length of life for lots of larvae fed respectively on various concentrations of galactose and on water

Lot No.	Food	Number of larvae	Weight of each	Length of life
			Mgm.	Hours
94	5 per cent galactose.....	20	19	6.8±0.19
95	15 per cent galactose.....	20	18	8.6±1.86
96	25 per cent galactose.....	20	19	11.38±.35
97	35 per cent galactose.....	20	20	13.51±1.26
98	50 per cent galactose.....	20	17	16.02±.91
99	Water.....	20	17	8.40±.44

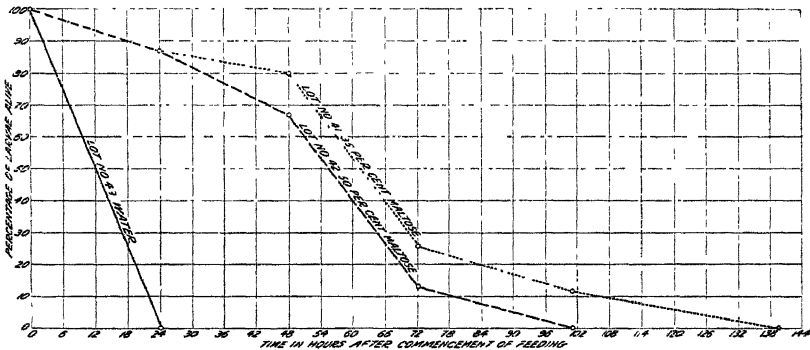


FIG. 5.—Percentages of survivors of lots of honeybee larvae, at intervals after the commencement of feeding, fed on solutions of maltose in certain concentrations with water, and of one lot supplied with water only

TABLE 8.—Observations to ascertain the relative duration of life of representative lots of honeybee larvae fed on sucrose in specified concentrations with water, and lots supplied with water only, as checks<sup>a</sup>

Lot 35 (25 per cent)		Lot 36 (35 per cent)		Lot 37 <sup>b</sup> (water)		Lot 83 (35 per cent)		Lot 93 <sup>c</sup> (water)	
Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving
Hours	Per cent	Hours	Per cent	Hours	Per cent	Hours	Per cent	Hours	Per cent
5.5	100	5.5	100	5.5	100	6.0	95	3.5	95
23.0	90	22.5	90	22.5	30	11.5	95	16.0	35
29.5	90	29.0	90	29.0	30	18.0	95	16.0	0
47.0	60	45.5	80	45.5	0	24.0	90		
53.5	60	53.5	70			30.0	90		
70.5	60	70.0	70			35.5	90		
77.0	50	77.0	70			42.0	90		
94.5	40	94.0	40			48.0	75		
102.0	30	101.5	40			54.0	55		
118.5	10	118.0	40			59.5	30		
129.5	0	129.0	30			66.0	20		
		166.0	10			72.0	10		
		190.0	0			78.0	10		
						84.0	5		
						90.0	0		

<sup>a</sup> Data presented graphically in fig. 4.  
<sup>b</sup> Check on lots 35 and 36.  
<sup>c</sup> Check on lot 83.

TABLE 9.—Average length of life for lots of larvae fed respectively on solutions of sucrose and on water

Lot No.	Food	Number of larvae	Weight of each	Length of life
			<i>Mgm.</i>	<i>Hours</i>
34	15 per cent sucrose .....	10	42	45.20± 3.45
35	25 per cent sucrose .....	10	44	73.12± 8.15
36	35 per cent sucrose .....	10	41	95.50±11.30
37	Water (check on lots 34, 35, 36) .....	10	45	21.27± 2.48
80	5 per cent sucrose .....	20	16	11.80± .62
81	15 per cent sucrose .....	20	17	17.98± 1.18
82	25 per cent sucrose .....	20	18	32.90± 2.95
83	35 per cent sucrose .....	20	18	54.00± 2.82
84	50 per cent sucrose .....	20	19	59.60± 4.23
93	Water (check on lots 80, 81, 82, 83, 84) .....	20	18	8.68± .15
125	75 per cent sucrose .....	20	17	68.85± 7.11
122	100 per cent sucrose .....	20	14	52.28± 3.47
129	Water (check on lots 125, 122) .....	20	17	4.61± .23

TABLE 10.—Observations to ascertain the relative duration of life of representative lots of honeybee larvae fed on maltose in specified concentrations with water, and a lot supplied with water only, as a check\*

Lot 41 (35 per cent maltose)		Lot 42 (50 per cent maltose)		Lot 43 (water)	
Time	Surviving	Time	Surviving	Time	Surviving
<i>Hours</i>	<i>Per cent</i>	<i>Hours</i>	<i>Per cent</i>	<i>Hours</i>	<i>Per cent</i>
24.0	87	24.0	87	24	0
48.5	80	48.5	67	-----	-----
72.0	27	72.0	13	-----	-----
101.0	13	101.0	0	-----	-----
139.5	0	-----	-----	-----	-----

\* Data presented graphically in fig. 5.

TABLE 11.—Average length of life for lots of larvae fed respectively on solutions of maltose and on water

Lot No.	Food	Number of larvae	Weight of each	Length of life
			<i>Mgm.</i>	<i>Hours</i>
38	5 per cent maltose .....	15	18	15.86±1.16
39	15 per cent maltose .....	15	24	60.70±5.13 ✓
40	25 per cent maltose .....	15	19	46.95±2.63
41	35 per cent maltose .....	15	22	63.71±4.78
42	50 per cent maltose .....	15	21	52.51±3.92
43	Water (check on lots 38, 39, 40, 41, 42) .....	15	23	12 (between 0 and 24)
85	5 per cent maltose .....	20	19	11.20± .46
86	15 per cent maltose .....	20	18	18.40±1.31
87	25 per cent maltose .....	18	19	23.00±1.47
88	35 per cent maltose .....	18	17	28.06±2.90
89	50 per cent maltose .....	20	14	13.87±1.24
93	Water (check on lots 85, 86, 87, 88, 89) .....	20	18	8.68± .15

The lots fed on lactose were run in two series. In the first series the greatest age was reached by lot 51 (Tables 12 and 13), but since the first examination was not made until 20 hours after the beginning of the experiment, and all the larvae in water were dead, as shown in Figure 6, A, it was impossible to give the length of life of the water check of the first series, lot 52, except as between 0 and 20 hours. For this reason another series of lots was run with examinations at

6-hour intervals. (Fig. 6, B.) In this series all lots showed a longer life on lactose than on water, lot 92, on a saturated solution of lactose,

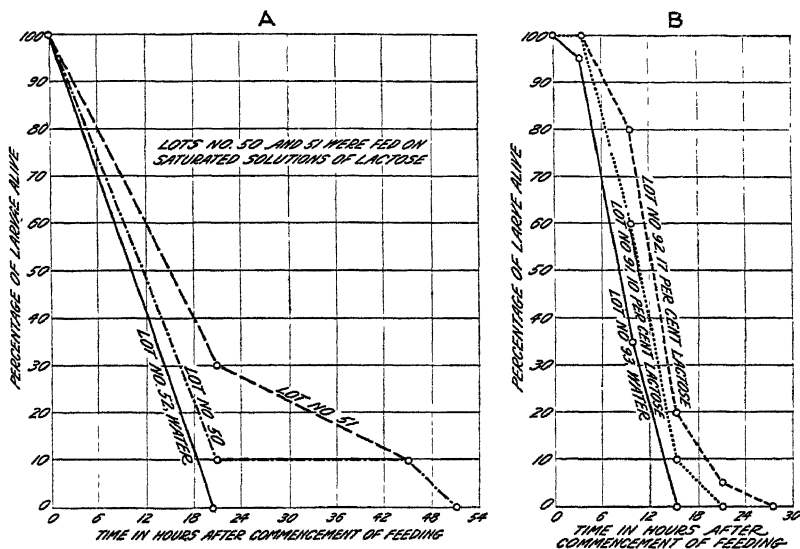


FIG. 6.—Percentages of survivors of lots of honeybee larvae, at intervals after the commencement of feeding, fed on solutions of lactose in certain concentrations with water, and of lots supplied with water only, as checks. A, two lots, fed on saturated solutions of lactose, and one, supplied with water only; all the larvae of this lot were found to have died in the first interval of 20 hours. B, two lots, fed on lactose in different concentrations, and one, supplied with water only.

living  $13.18 \pm 0.66$  hours, as compared with  $8.68 \pm 0.15$  hours for the water check, lot 93, a ratio of about  $1\frac{1}{2}$  to 1. This seems to indicate that the larvae are able to digest lactose.

TABLE 12.—Observations to ascertain the relative duration of life of representative lots of honeybee larvae fed on lactose in specified concentrations with water, and lots supplied with water only, as checks<sup>a</sup>

Lot 50 (saturated solution)		Lot 51 (saturated solution)		Lot 52 <sup>b</sup> (water)		Lot 91 (10 per cent)		Lot 92 (17 per cent)		Lot 93 <sup>c</sup> (water)	
Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving
Hours	Per cent	Hours	Per cent	Hours	Per cent	Hours	Per cent	Hours	Per cent	Hours	Per cent
20.5	10	21.0	30	20.5	0	4.0	100	3.5	100	3.5	95
44.5	10	44.0	10	-----	-----	10.5	60	10.0	80	10.0	35
51.0	0	51.0	0	-----	-----	16.5	10	16.0	20	16.0	0
-----	-----	-----	-----	-----	-----	22.0	0	21.5	5	-----	-----
-----	-----	-----	-----	-----	-----	-----	-----	27.5	0	-----	-----

<sup>a</sup> Data presented graphically in fig. 6.

<sup>b</sup> Check on lots 50 and 51.

<sup>c</sup> Check on lots 91 and 92.

TABLE 13.—Average length of life for lots of larvae fed respectively on solutions of lactose and on water

Lot No.	Food	Number of larvae	Weight of each	Length of life
			Mgm.	Hours
48	5 per cent lactose.....	10	17	{10 (between 0 and 20).
49	Saturated solution lactose.....	10	18	12.97±1.12
50	do.....	10	20	13.95±1.87
51	do.....	10	22	18.60±3.19
52	Water (check on lots 48, 49, 50, 51).....	9	20	{10 (between 0 and 20).
90	5 per cent lactose.....	20	15	11.62±.64
91	10 per cent lactose.....	20	16	11.57±.59
92	17 per cent lactose.....	20	15	13.18±.66
93	Water (check on lots 90, 91, 92).....	20	18	8.68±.15

The larvae fed on trehalose lived longer on all concentrations tested than they did on water. A comparison of the lengths of life of lot 112 and lot 121 (Tables 14 and 15 and fig. 7) gives a ratio of approximately 3 to 1. It appears, then, that the larvae, like the

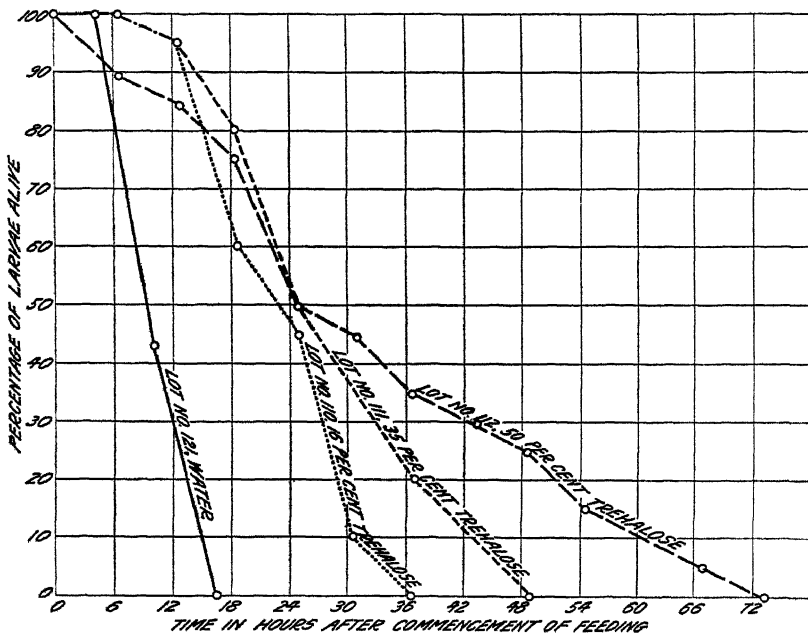


FIG. 7.—Percentages of survivors of lots of honeybee larvae, at intervals after the commencement of feeding, fed on solutions of trehalose in certain concentrations with water, and of one lot supplied with water only

adults, can digest this rare sugar with considerable readiness, although it has probably never been a constituent of their diet.

It was found, then, that bee larvae can digest all the disaccharides tested, and that in ability to sustain life these appear to rank in the order sucrose, maltose, trehalose, and lactose.

TABLE 14.—*Observations to ascertain the relative duration of life of representative lots of honeybee larvae fed on trehalose in specified concentrations with water, and one lot supplied with water only, as a check*<sup>a</sup>

Lot 110 (16 per cent)		Lot 111 (35 per cent)		Lot 112 (50 per cent)		Lot 121 (water)	
Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving
<i>Hours</i>	<i>Per cent</i>	<i>Hours</i>	<i>Per cent</i>	<i>Hours</i>	<i>Per cent</i>	<i>Hours</i>	<i>Per cent</i>
7.0	100	7.0	100	7.0	90	4.5	100
13.5	95	13.5	95	13.5	85	10.5	43
19.5	60	19.5	80	19.5	75	17.0	0
25.5	45	25.5	50	25.5	50	-----	-----
31.0	10	31.0	35	31.0	45	-----	-----
37.0	0	37.0	20	37.0	35	-----	-----
-----	-----	43.5	10	43.5	30	-----	-----
-----	-----	49.5	0	49.5	25	-----	-----
-----	-----	-----	-----	55.5	15	-----	-----
-----	-----	-----	-----	67.0	5	-----	-----
-----	-----	-----	-----	73.5	0	-----	-----

<sup>a</sup> Data presented graphically in fig. 7.TABLE 15.—*Average length of life for lots of larvae fed respectively on solutions of trehalose and on water*

Lot No.	Food	Number of larvae	Weight of each	Length of life
			<i>Mgm.</i>	<i>Hours</i>
110	16 per cent trehalose.....	20	13	22.95±1.01
111	35 per cent trehalose.....	20	13	27.75±1.50
112	50 per cent trehalose.....	20	15	31.17±2.89
121	Water.....	23	13	10.21±.43

## TRISACCHARIDES

The only trisaccharide used in these experiments was melezitose, a sugar occurring in the sap of certain plants and in honeydew. The data indicate clearly that honeybee larvae are able to use melezitose as food, the length of life for lot 104, on 50 per cent melezitose, being about four and one-third times that of lot 109, on water. (Tables 16 and 17 and fig. 8.)

TABLE 16.—*Observations to ascertain the relative duration of life of representative lots of honeybee larvae fed on melezitose in specified concentrations with water, and one lot supplied with water only, as a check*<sup>a</sup>

Lot 102 (25 per cent)		Lot 103 (35 per cent)		Lot 104 (50 per cent)		Lot 109 (water)	
Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving
<i>Hours</i>	<i>Per cent</i>	<i>Hours</i>	<i>Per cent</i>	<i>Hours</i>	<i>Per cent</i>	<i>Hours</i>	<i>Per cent</i>
6.0	95	6.0	100	6.0	100	6.0	94
12.5	90	12.5	95	12.5	90	12.0	0
18.5	60	18.5	60	18.5	70	-----	-----
24.5	45	24.5	50	24.5	65	-----	-----
30.5	20	30.5	35	30.5	55	-----	-----
36.5	10	36.5	35	36.5	45	-----	-----
42.5	10	42.5	20	42.5	35	-----	-----
48.5	0	48.5	15	48.5	30	-----	-----
-----	-----	62.5	0	62.5	20	-----	-----
-----	-----	-----	-----	67.5	20	-----	-----
-----	-----	-----	-----	77.5	0	-----	-----

<sup>a</sup> Data presented graphically in fig. 8.

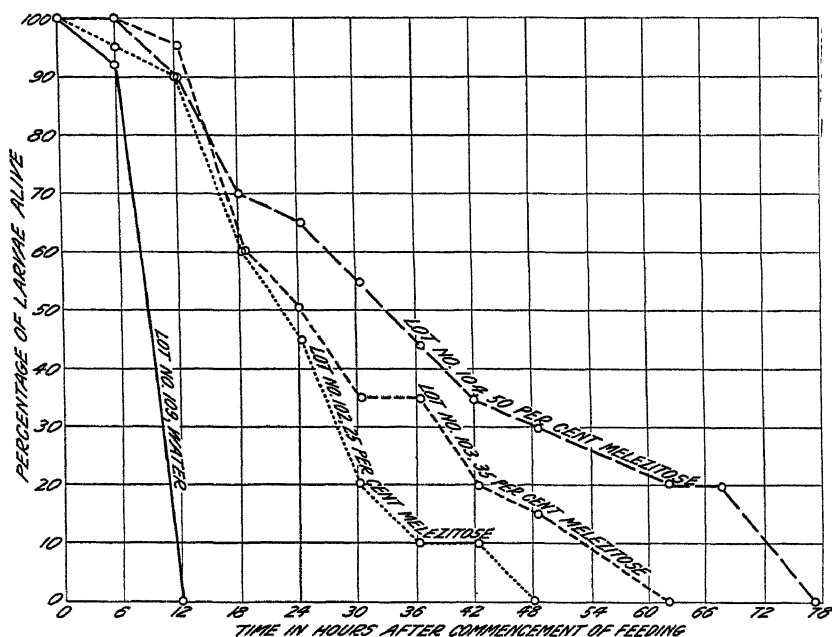


FIG. 8.—Percentages of survivors of lots of honeybee larvae, at intervals after the commencement of feeding, fed on solutions of melelitose in certain concentrations with water, and of one lot supplied with water only

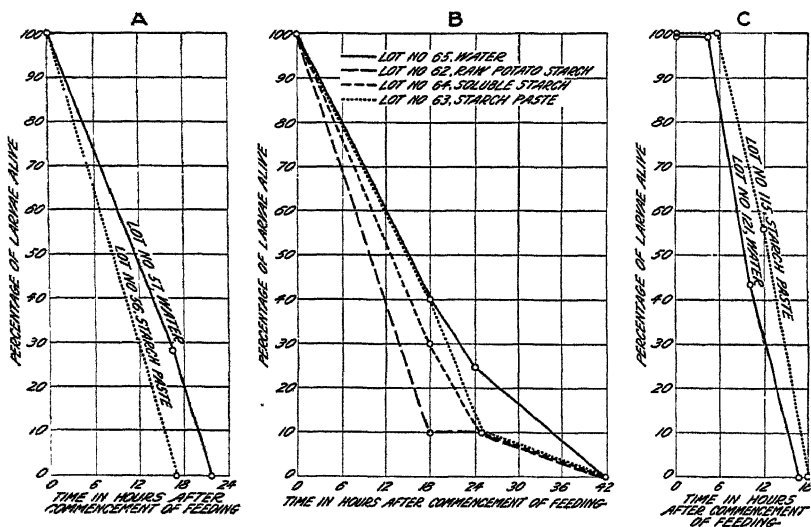


FIG. 9.—Percentages of survivors of lots of honeybee larvae, at intervals after the commencement of feeding, fed on combinations of starch and water, and others supplied with water only as checks. A, lot fed on starch paste, and lot supplied with water; B, three lots, fed respectively on raw potato starch, starch paste, and soluble starch, and one lot supplied with water only; C, one lot fed on starch paste, and one supplied with water only

TABLE 17.—Average length of life for lots of larvae fed respectively on solutions of melezitose and on water

Lot No.	Food	Number of larvae	Weight of each	Length of life
			Mgm.	Hours
100	5 per cent melezitose.....	20	11	9.25±0.28
101	15 per cent melezitose.....	20	11	16.30±1.05
102	25 per cent melezitose.....	20	11	23.26±2.56
103	35 per cent melezitose.....	20	11	28.58±2.24
104	50 per cent melezitose.....	20	11	37.47±3.31
109	Water.....	16	11	8.62±.24

POLYSACCHARIDES

The polysaccharides used in these experiments were dextrin, glycogen, and starch. The data obtained for these three carbohydrates show that of the five lots fed on starch only one (lot 115) lived longer than the corresponding water check, and even that one less than two hours longer. (Tables 18 and 20 and figs. 9 and 10.) There was no significant difference between the length of life of the lot fed on glycogen (lot 114) and its water check, lot 121. (Tables 18 and 20 and fig. 10.) The lot fed on dextrin (113), however, lived nearly three times as long as the corresponding lot (121) fed on water alone. (Tables 19 and 20 and fig. 10.)

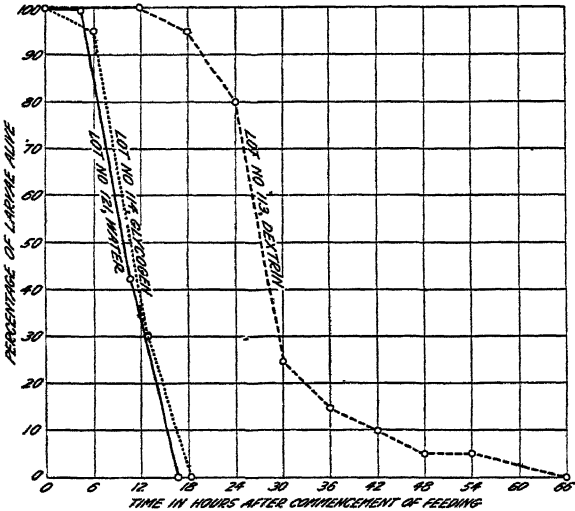


FIG. 10.—Percentages of survivors of three lots of honeybee larvae, at intervals after the commencement of feeding; one supplied with water only, as a check, and the others fed on solutions of dextrin and glycogen, respectively

TABLE 18.—Observations to ascertain the relative duration of life of representative lots of honeybee larvae fed on combinations of starch and water, and lots supplied with water only, as checks <sup>a</sup>

Lot 56 (starch paste)		Lot 57 <sup>b</sup> (water)		Lot 62 (raw starch)		Lot 63 (starch paste)		Lot 64 (soluble starch)		Lot 65 <sup>c</sup> (water)		Lot 115 (starch paste)		Lot 121 <sup>d</sup> (water)	
Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving
Hrs.	P. ct.	Hrs.	P. ct.	Hrs.	P. ct.	Hrs.	P. ct.	Hrs.	P. ct.	Hrs.	P. ct.	Hrs.	P. ct.	Hrs.	P. ct.
17	0	16.5	29	18.0	10	18.0	40	18.0	30	18.0	40	5.5	100	4.5	100
-----	-----	22.0	0	24.5	10	24.5	10	24.5	10	24.5	25	12.0	55	10.5	43
-----	-----	-----	-----	41.5	0	41.5	0	41.5	0	41.5	0	18.0	0	17.0	0

<sup>a</sup> Data presented graphically in fig. 9. <sup>c</sup> Check on lots 62, 63, and 64. <sup>b</sup> Check on lot 56. <sup>d</sup> Check on lot 115.

The results indicate that the larvae are able to utilize dextrin, but neither glycogen nor starch.

It should be added here that an examination of larvae which had been floating on a starch mixture for a few hours showed countless grains of starch in the intestine, and therefore the failure to gain nourishment from starch was not due to a failure to take the starch into the alimentary tract.

TABLE 19.—*Observations to ascertain the relative duration of life of three lots of honeybee larvae, one supplied with water only, as a check, and the others fed on solutions of dextrin and glycogen, respectively*<sup>a</sup>

Lot 113 (30 per cent dextrin)		Lot 114 (50 per cent glycogen)		Lot 121 (water)	
Time	Surviving	Time	Surviving	Time	Surviving
<i>Hours</i>	<i>Per cent</i>	<i>Hours</i>	<i>Per cent</i>	<i>Hours</i>	<i>Per cent</i>
6.0	100	6.0	95	4.5	100
12.5	100	12.5	30	10.5	43
18.5	95	18.5	0	17.0	0
24.5	80				
30.0	25				
36.0	15				
42.5	10				
48.5	5				
54.5	5				
66.5	0				

<sup>a</sup> Data presented graphically in fig. 10

TABLE 20.—*Average length of life for lots of larvae fed respectively on starch, dextrin, glycogen, and water*

Lot No.	Food	Number of larvae	Weight of each	Length of life
			<i>Mgm.</i>	<i>Hours</i>
56	Starch paste.....	10	23	8.5 (between 0 and 17)
57	Water (check on lot 56).....	7	23	
62	Raw starch.....	20	43	11.40±.85
63	Starch paste.....	20	42	15.07±1.41
64	Soluble starch.....	20	46	13.85±1.32
65	Water (check on lots 62, 63, 64).....	20	42	16.83±1.91
115	Starch paste.....	20	15	12.18±.46
114	50 per cent glycogen.....	20	12	10.81±.50
113	30 per cent dextrin.....	20	15	29.55±1.24
121	Water (check on lots 115, 114, 113).....	23	13	10.21±.43

#### HONEYS

A study of the ability of honeybee larvae to utilize carbohydrates as food would, of course, be incomplete without an investigation of their relative ability to use honey, which is the main carbohydrate constituent of their normal diet. Bees gather nectar from many sources, and from the different nectars make honeys varying considerably in physical and chemical characteristics. Since, as is well known among beekeepers, these differ in value as food for adult bees, it is probable that they also differ in value as food for the brood.

In order to investigate this question, several lots of larvae were fed on honeys differing as widely as possible in source, color, odor, taste, dextrin content, dextrose-levulose ratio, tannin content, sucrose con-

tent, and the like. These honeys were from sage, white clover, alfalfa, tulip tree, aster, goldenrod, buckwheat, wild buckwheat, mountain laurel, locust, and basswood clover, the latter consisting of three parts of basswood to one of clover. At the same time, for check and comparison, lots were also fed on distilled water and on 75 per cent cane sugar.

The average length of life of the lots fed on the various honeys ranges from  $59.82 \pm 3.89$  hours on basswood clover to  $12.39 \pm 0.84$  hours on locust. (Table 22.) All the honeys tested may be arranged in the following order from greatest to least length of life: Basswood clover, white clover, tulip tree, alfalfa, buckwheat, mountain laurel (poisonous to humans), sage, wild buckwheat, aster, goldenrod, and locust. The average for the 11 honeys tried is 38.93 hours (probable error disregarded), which is essentially the same as for lot 138, fed on 75 per cent cane sugar, and is approximately three and one-half times as long as for lot 139, fed on water alone. (Tables 21 and 22 and fig. 11.)

TABLE 21.—Observations to ascertain the relative duration of life of representative lots of honeybee larvae; one fed on 75 per cent solution of cane sugar, one on locust honey, one on a mixture of 75 per cent of basswood honey and 25 per cent of clover honey, and one supplied with water only as a check <sup>a</sup>

Lot 140 (75 per cent basswood honey, 25 per cent clover honey)		Lot 142 (locust honey)		Lot 138 (75 per cent cane sugar)		Lot 139 (water)	
Time	Surviving	Time	Surviving	Time	Surviving	Time	Surviving
Hours	Per cent	Hours	Per cent	Hours	Per cent	Hours	Per cent
5.0	100	4.5	96	5.0	96	5.0	100
16.5	96	16.0	16	16.5	72	16.5	0
23.0	96	22.5	12	23.0	72		
28.5	96	28.0	8	28.5	68		
33.5	84	33.0	4	33.5	68		
41.0	68	40.5	0	41.0	44		
47.0	64			47.0	32		
53.0	48			53.0	28		
65.0	44			65.0	20		
71.0	32			71.0	16		
77.0	28			77.0	8		
89.0	20			89.0	4		
95.0	12			95.0	4		
101.0	8			101.0	4		
113.0	0			113.0	0		

<sup>a</sup> Data presented graphically in fig. 11.

These data show no correlation between length of life of the larvae and any known physical or chemical characteristics of the honey. For example, the length of life on the light honeys, white clover, alfalfa, sage, locust, and basswood clover, averaged 41+ hours, which is not significantly different from the average of 39+ hours on the darker honeys, tulip tree, buckwheat, wild buckwheat, and aster; the tulip-tree honey, with high dextrin content,<sup>5</sup> sustained life as long as sage, clover, or alfalfa, with low dextrin content; tulip tree and buckwheat, strong in flavor and odor and giving strong tannin reactions, had about the same effect as the milder

<sup>5</sup> For chemical analysis of honeys, see Browne (6).

sage and basswood clover, having very little tannin; sago, with a high dextrose-levulose ratio, was practically equal in life-sustaining value to alfalfa, with low dextrose-levulose ratio.

TABLE 22.—Average length of life for lots of larvae fed respectively on various honeys, cane sugar, and water

Lot No.	Food	Number of larvae	Length of life
			Hours
130	Sage honey.....	25	39.44±3.09
131	White clover honey.....	25	51.23±3.69
132	Alfalfa honey.....	25	45.39±4.13
133	Tulip-tree honey.....	25	50.16±3.25
134	Aster honey.....	25	24.79±2.09
135	Goldenrod honey.....	25	18.72±1.69
136	Buckwheat honey.....	25	44.57±2.40
137	Wild buckwheat honey.....	25	38.45±2.64
140	Basswood honey, 75 per cent; clover honey, 25 per cent.....	25	59.82±3.89
141	Mountain laurel honey (poison).....	25	43.30±1.74
142	Locust honey.....	25	12.89±.84
	Average of all honeys.....		38.93
138	75 per cent cane sugar.....	25	40.86±3.53
139	Water.....	25	10.70

• The larvae were not weighed, but all lots are comparable as to size and age.

It is a fact, however, that not all honeys were of equal value in sustaining life. This fact may have been due to an unknown characteristic of the honeys, to an uncontrolled factor in the experiment, or to a difference in the larvae themselves.

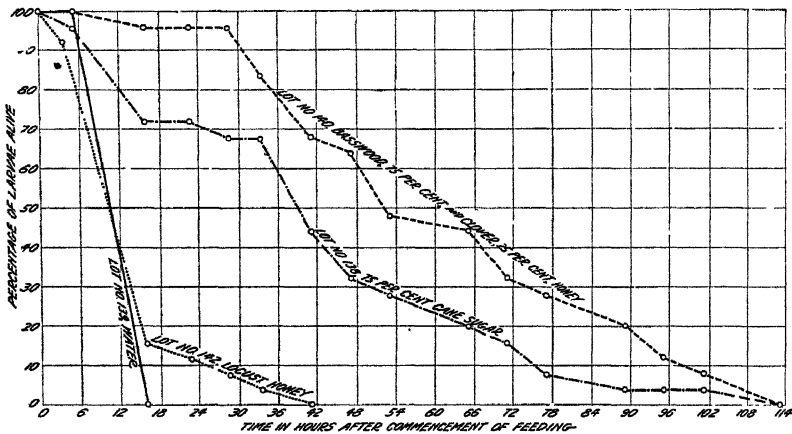


FIG. 11.—Percentages of survivors of lots of honeybee larvae, at intervals after the commencement of feeding; one fed on a 75 per cent solution of cane sugar, one on locust honey, one on a mixture of 75 per cent of basswood honey and 25 per cent of clover honey, and one supplied with water only.

REVIEW AND COMPARISON

In the preceding pages have been presented in detail the results obtained in experiments on honeybee larvae fed on solutions of the monosaccharides, dextrose, levulose, and galactose; of the disaccharides, sucrose, maltose, lactose, and trehalose; of the trisaccharide, melezitose; and of the polysaccharides, dextrin, starch, and

glycogen; and on various honeys. A comparison of the life-sustaining value of these various carbohydrates can now readily be made by first computing a ratio for each carbohydrate and then comparing the ratios. The ratio in each case is found by dividing the length of life of the optimum lot in each series by the length of life of its particular water check. In case more than one series of lots were run for a single carbohydrate, an average is taken of the ratios of all the series for that carbohydrate. (Table 23.) A ratio of one, or less than one, means, of course, that the length of life on that carbohydrate was equal to or less than the length of life on water, and indicates no food value at all.

TABLE 23.—*Comparison of various carbohydrates as to their value in sustaining the life of honeybee larvae. All carbohydrates tried appear to have food value except starch and glycogen (probable error disregarded)*

Name	Carbohydrate (=C)		Starvation check (water) (=S)		Ratio (C/S)	Average ratio (C/S)
	Lot No	Length of life	Lot No.	Length of life		
Sucrose	36	95.50	37	21.27	4.5	
Do.	84	59.66	93	8.68	6.9	
Do.	125	68.55	120	4.61	14.9	
Do.	138	40.86	139	10.70	3.8	7.5
Levulose	79	47.56	73	10.10	4.7	4.7
Maltose	41	63.71	43	12.00	5.3	
Do.	88	28.06	93	8.68	3.2	4.3
Melezitose	104	37.47	109	8.62	4.3	4.3
Dextrose	75	39.90	73	10.10	4.0	4.0
Honeys	( <sup>a</sup> )	38.93	139	10.70	3.6	3.6
Trehalose	112	31.17	121	10.21	3.1	3.1
Dextrin	113	29.55	121	10.21	2.9	2.9
Galactose	98	16.02	99	8.40	1.9	1.9
Lactose	51	18.60	52	10.00	1.9	
Do.	92	13.18	93	8.68	1.5	1.7
Starch	56	8.50	47	11.89	.7	
Do.	63	15.07	65	16.33	.9	
Do.	115	12.18	121	10.21	1.2	.9
Glycogen	114	10.81	121	10.21	1.1	1.1

<sup>a</sup> Average.

These data show (Table 23) that sucrose, with a ratio of 7.5, had the greatest life-sustaining value, and that following this came, in order, levulose (4.7), maltose (4.3), melezitose (4.3), dextrose (4.0), honeys (3.6), trehalose (3.1), dextrin (2.9), galactose (1.9), lactose (1.7), glycogen (1.1), and starch (0.9). Glycogen and starch, therefore, had no more food value than distilled water.

## ENZYMES

Practically all work done on digestion in insects has been a study of the various enzymes produced in the insect, either in the body as a whole or in some particular part of it. If the results obtained by feeding are to be compared with results obtained by investigating the enzymes, it follows that both must be reduced to common terms.

Our knowledge of digestion in general indicates that an organism which is able to get nourishment from a carbohydrate other than a monosaccharide must first change this carbohydrate to a monosaccharide. Likewise, our knowledge of enzymes indicates that a specific enzyme is necessary for the hydrolysis of each complex carbohydrate

to a simpler form. It therefore seems clear that if an insect is able to obtain nourishment from a complex carbohydrate it must produce the specific enzyme necessary to hydrolyze this carbohydrate, and it must secrete this enzyme at a place where it may be in contact with the food.

To state the results of these experiments in terms of enzymes, it seems clear that the honeybee larva has available for use in digestion, the following enzymes: Invertase, maltase, melezitase, trehalase, dextrinase, and lactase, but does not have available any diastase.

The fact that there is no diastase available for use in digestion was quite unexpected, in view of the apparent widespread distribution of diastase among insects and its presence even in the adult honeybee, as reported by Erlenmeyer, Axenfeld, Petersen, and Pavlovsky and Zarin. (Table 1.) But is it not possible for diastase to be produced in an insect and still not be secreted into the intestine?

In order to obtain direct evidence concerning this question a number of larvae were punctured and their coelomic fluid (blood) collected. A few cubic centimeters of this fluid were added to a thin starch paste and incubated overnight at a temperature of 37.5 °C. The next morning the starch had disappeared and the solution gave a distinct reaction to Fehling's test for sugar, thus showing clearly that diastase is present in the coelomic fluid. If, now, it be recalled that, as before mentioned, the larvae take starch into their alimentary tract, but receive no nourishment from it, it seems evident that although diastase is present in the larva as a whole, it is not present in the lumen of its digestive tract. This conclusion is essentially the same as that of Phillips (17) for the adult honeybee.

At the same time, tests were made to ascertain whether or not invertase, maltase, and lactase were present in the coelomic fluid. Coelomic fluid was added to solutions of pure sucrose, maltose, and lactose, and the hydrogen-ion concentration was adjusted to  $P_H$  6.8. Then the specific rotation of the solution was ascertained by means of the polariscope, after which the solution was covered with toluol and allowed to stand for 24 hours. The polariscope reading was then again taken but in no case was any significant change noted. Such negative results give an indication, although by no means a proof, that neither invertase, maltase, nor lactase is present in the coelomic fluid of the larva, although from feeding experiments these enzymes are shown to be available for use in digestion.

#### SUMMARY AND CONCLUSIONS

Honeybee larvae may be removed from the comb and kept alive under artificial conditions such as are described under the head, "Material and method."

They are able to utilize as food the following carbohydrates, named in the order of their apparent food value: Sucrose, levulose, maltose, melezitose, dextrose, trehalose, dextrin, galactose, and lactose (the last very slightly). This fact indicates that the larva has available the digestive enzymes invertase, maltase, melezitase, trehalase, dextrinase, and lactase. Honeybee larvae apparently differ from adult honeybees, then, in their ability to use dextrin and galactose (according to Phillips) and lactose (according to both Phillips and Pavlovsky and Zarin). Lactase, has, however, been

found in the larvae of *Euproctis chrysorrhoea*, *Bombyx mori*, *Hyponomeuta* sp., and *Tenebrio molitor*. (Table 1.)

Two carbohydrates which honeybee larvae are not able to use as food are starch and glycogen. The enzyme diastase is therefore not present in the lumen of the gut. But the enzyme diastase is present in the coelomic fluid of the larvae, as shown by the ability of the coelomic fluid to digest starch. In this respect, according to Phillips, the larvae are like the adult honeybees; according to all other investigators they are unlike the adults. They are also apparently unlike almost every other insect which has been investigated.

Larvae are like adults in their ability to metabolize two rare sugars, melezitose and trehalose. Their ability to use trehalose is especially remarkable in view of the fact that this sugar probably never occurs in their normal diet.

Under the conditions of these experiments honeys show a life-sustaining value slightly less than sucrose, and approximately equal to levulose, maltose, melezitose, and dextrose. The various honeys used ranked in the following order from greatest to least life-sustaining value: Basswood clover, white clover, tulip tree, alfalfa, buckwheat, mountain laurel, sage, wild buckwheat, aster, goldenrod, and locust; the reason for this order of values is not clear, since it is not to be explained on the basis of any known physical or chemical characteristic of the honeys.

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# A ROOT ROT OF LUCRETIA DEWBERRY CAUSED BY A VARIETY OF COLLYBIA DRYOPHILA FR.<sup>1</sup>

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## INTRODUCTION

The culture of the Lucretia dewberry *Rubus procumbens* Muhl. var. *roribaccus* Bailey was begun as a commercial enterprise about 25 years ago in the sand hills, where it is planted from Sanford to Hamlet in North Carolina. Although the industry has increased rapidly, the plant in many fields has lately become badly diseased and at times unprofitable (11).<sup>2</sup> The most recent trouble is a root rot that is certain to become a much greater economic factor in growing berries because it severely reduces the productiveness of the plants, and by causing the death of some plants leaves a ragged irregular stand, which has not been corrected by cultural practices. It has been necessary to remove plants affected by root rot and the loss has become of such economic importance that studies were begun for the purpose of determining the cause and its control. These studies have revealed an undescribed disease of the dewberry and a causal fungus heretofore unknown as a plant parasite.

The symptoms of the disease, the life cycle of the causal organism, its dissemination, and parasitism are described.

## HISTORY AND DISTRIBUTION

The root-rot disease was called to the attention of G. W. Fant, extension plant pathologist, during the growing season of 1922. The first reports were from Cameron, but investigations in 1926 revealed the fact that the disease was widespread throughout the sand hills, where it is apparently becoming worse. It is causing greater damage in some areas than in others; in some fields from 15 to 90 per cent of the plants are diseased. In the vicinity of Cameron the infection since 1922 has been so severe in some fields that all plants have been plowed out because of a diseased, unprofitable condition and the infected soil has since that time been used for other crops such as cotton and tobacco, which do not seem to be affected.

The origin of the disease is not known but may easily be the present dewberry fields, since there is no report of this disease occurring elsewhere. Coker and his associates (2) have collected sporophores from various habitats in many localities of the State that are very similar to the fruiting bodies of the causal organism. Furthermore, the same investigators have found sporophores very similar to the causal organism in oak and pine woods, which make up the natural vegetation of a greater portion of the sand hills. However, there are no acceptable data to show that the fungus has been a parasite on the previous vegetation of lands now used for planting the dewberry.

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<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 464.

While the disease appears to be confined to the sand hills in North Carolina, other varieties of the causal fungus are widely distributed geographically according to Buller (1, p. 465, 470), who says: "*Collybia dryophila* is a well-known agaric commonly found in woods, both in Europe and North America. . . . In English woods, especially those of Sutton Park, near Birmingham, in the course of 20 years I must have seen some thousands of *C. dryophila*." McIlvaine (6) reported it from West Virginia, North Carolina, New Jersey, and Pennsylvania. In the latter State he reported a very pretty form, which was found on pine needles at Eagles Mere. Kauffman (4) found it very common throughout the State of Michigan, where the typical forms were in hardwood forests. There are slight differences



FIG. 1.—A, Field of Lucretia dewberries badly infected with root-rot disease (July 15): a, wilted canes on diseased plant; b, empty hill, plant set in which was killed by the disease. B, field of Lucretia dewberries August 15 after old canes were removed, showing: a, weak plants due to diseased roots; b, where plant was killed by the disease. C, Field of Lucretia dewberries July 15, showing healthy growth of canes on uninfected roots. D, Diseased canes, showing: a, girdling of cane; b, death of cane due to complete infection of all parts below; c, cane spur left after pruning, through which infection is common; d, small section of live tissue supporting weak cane growth above; e, small and bushy leaves

noted in descriptions of the *C. dryophila* by these authors, which are obvious, since McIlvaine (6, p. 121) says: "*C. dryophila* is so common and variable that descriptions would fail to cover it in its eccentricities," and Kauffman (4, p. 755) speaks of the *C. dryophila* "group" as "a whole series of variations," and he further believes that its varieties appear under different influences of habitat. This remarkable history of the fungus indicates that a parasitic variety could easily be confined to isolated areas without being distributed as widely as other varieties of *C. dryophila*.

The fungus is carried on roots of new plants, this being determined by isolating the fungus from plants developed in infected soils and by inoculating new plants. The white to tan colored rhizomorphs are sometimes prominent on the plants grown on infected soils. The

fungus is easily disseminated from one plant to another in the same field on pruning shears, which are used in July to cut out all canes to or just beneath the soil. It is quite obvious that the inoculum is disseminated in other ways in which the soil is disturbed.

#### SYMPTOMS OF DISEASE

To the casual observer the most obvious symptom is the death of plants following severe infection (fig. 1, A and B), and the ragged, irregular growth of canes in comparison to healthy plants (fig. 1, C). This is not the first nor the most significant symptom, however, since the plant may be severely stunted and may produce a very inadequate growth of canes and yield only a small quantity of berries for one or many seasons before death occurs. (Fig. 2.) The causal organism is confined to the parts of the plant at or below the soil level, where it produces an abundance of mycelium, which girdles the canes (fig. 1, D) and finally penetrates the tissues. The girdling is most severe on spring wood (fig. 3, A), but occurs also on the summer and fall wood (fig. 3, B). At this time the roots are also severely attacked and girdled by the fungus (fig. 4, A) and the separated parts are destroyed by an abundant growth of the fungus (fig. 4, B). When badly infected, the normal growth of the plant is arrested and wilting and sudden death are the results. (Fig. 3, B.) This condition may occur when the canes are young or, as is often the case, just as the berries are beginning to ripen. In the latter event the berries are inferior in quality or totally worthless. (Fig. 5.) The plants have a natural inclination to resist the disease by the production of new canes from the roots that extend below the infected parts. When the original stump is killed, a new one develops, and when this is killed, still another is produced. The process of death and production of new canes is often repeated a number of times, so that single plants having several dead stumps are often found. (Fig. 6, A.) Severely diseased stumps are unable to properly nourish the canes; consequently the latter remain short and bushy and may readily be distinguished by contrast with the more vigorous, more productive canes produced on healthy plants. (Fig. 2.)



FIG. 2.—Healthy plants on left, right, and in background; diseased plants with short canegrowth in the foreground

The foliage on the diseased canes is somewhat crowded, small, and has a purplish hue (fig. 1, D) in contrast to the green on the healthy plants.

When the stumps, roots, and canes of diseased plants are examined, rhizomorphs, mycelium, and wefts of white, fungous hyphae of the causal fungus are easily found throughout the year. (Fig. 6, B.) When the fungus has lived on the same rootstock for several years, the weft of fungous hyphae in the bark and cambium become very pronounced. (Fig. 6, C.) The fungus parts are white or some shade of tan or brown and the wood rot is noticeable also after the fungus enters the old spurs and penetrates downward into the live



FIG. 3.—A, Plants inoculated with a pure culture of the causal organism in August, 1926, showing: a, mycelium and rhizomorphs on 1926 cane in April; b, fungus on 1927 cane in April. Formed in March, many of these canes were killed before May 1, 1927. B, Diseased plants, showing: a, sudden wilting due to complete infection of stem and parts of root system; b, uninfected stem and root system; c, infection of spur at surface of soil, where wilting and death of several canes in the center of this plant is due to the killing of the tissues between canes and stem by the disease.

wood (fig. 6, D), causing its destruction (fig. 7, A). At this stage the strands of hyaline mycelium are present in the xylem, sometimes almost completely filling the cell. (Fig. 7, B.)

#### THE CAUSAL ORGANISM AND ITS LIFE HISTORY

The disease is caused by a parasitic variety or strain of *Collybia dryophila* Fr. This fungus lives over in and on the tissues of the infected roots and canes in the soil, and there it remains active throughout the winter months, as well as during the growing season. The fact that the fungus was isolated from infected parts early in March, and at other periods throughout the year, is definite proof that the fungus parts are not destroyed by high and low temperatures prevailing in this area during the summer and winter months. The hibernating of the mycelium in the tissues is certain to maintain the life cycle of the fungus for a few if not for many years without the

aid of the spores, since no sporophores were found on dewberry plants in the field during 1926, which was probably due to a severe summer drought. In spite of this fact the fungus was isolated from diseased plants collected in the field in the early part of March of both the 1926 and 1927 seasons.

The fungus is readily obtained in pure culture by plating on prune agar in the usual manner strands of mycelium from rhizomorphs taken from live roots, to escape saprophytes on dead tissues. The prune medium is composed of 10 grams of agar, 125 grams of large prunes, and 1 liter of water. On this medium the mycelium is nearly pure white for 30 days, and sometimes longer, finally becoming creamish and shades of tan. Although the fungus is isolated with some difficulty when neutral media of the ordinary strength of plant coccations and synthetic combinations are used, it grows on these media when large amounts of inoculum are employed or when made slightly acid. The mycelium grows well on rice, potato, sweet potato, apple, corn meal, and Cook's synthetic medium. The mycelium development in barnyard manure is especially well suited for inoculation tests because of the ease with which the inoculum is removed from culture flasks and placed around small roots and stems. While the growth of the mycelium is very abundant on a large number of media, the sporophores have been obtained on parts of the dewberry plant only. This medium was prepared by cutting sections of roots and canes so that the parts could be easily inserted into 2-liter flasks. The medium was moistened, sterilized in the usual manner, and held for 15 days to assure complete sterilization, after which a pure culture of the fungus was added. The culture was incubated at 25° C. in candle light. In 48 hours the growth of the mycelium was evident, and in another 48 hours the rhizomorphs were forming in all parts of the flask. Sporophores began to develop in 30 days, but on the

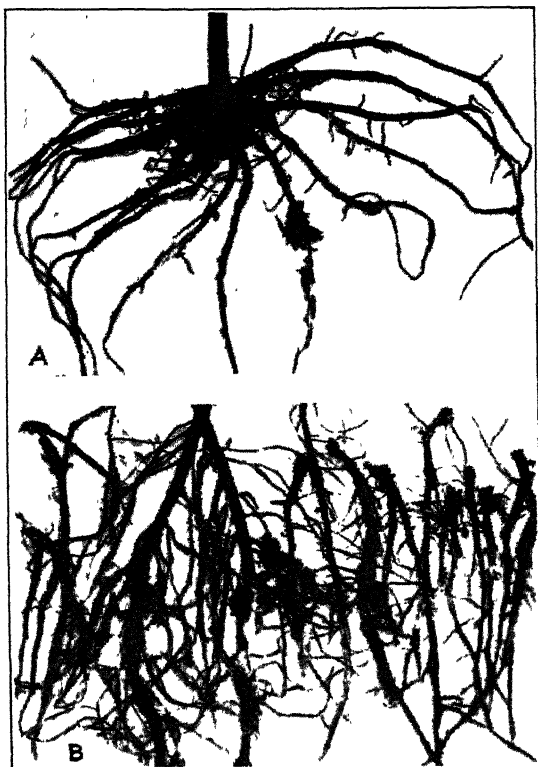


FIG. 4.—A, Results of inoculating new plants. Lesions shown on diseased roots and white mycelium developed abundantly 30 days after inoculation. B, Diseased roots taken from inoculated plants, showing lesions, destruction of roots, and the prolific growth of mycelium

dewberry parts alone they developed slowly and were not always well developed. When prune medium was added to the dewberry parts, it

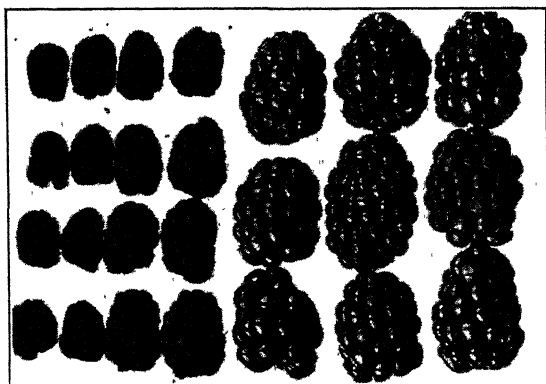


FIG. 5.—Normally ripened berries from uninfected canes compared with those obtained from wilted canes on the same plant as that shown in fig. 3, B, b

increased the growth of mycelium, rhizomorphs, and sporophores. The color of the mycelium and rhizomorph on plants in the field and in cultures in the laboratory is similar in every way. The development of the mycelium and rhizomorphs has much to do with the production of sporophores, since the largest number of sporophores were obtained when the rhizomorphs were most abundant. Sterile

water was added to all flasks at frequent intervals in order to extend the growth of the mycelium and rhizomorphs and thus to determine the duration of sporophore production on the medium used. In these

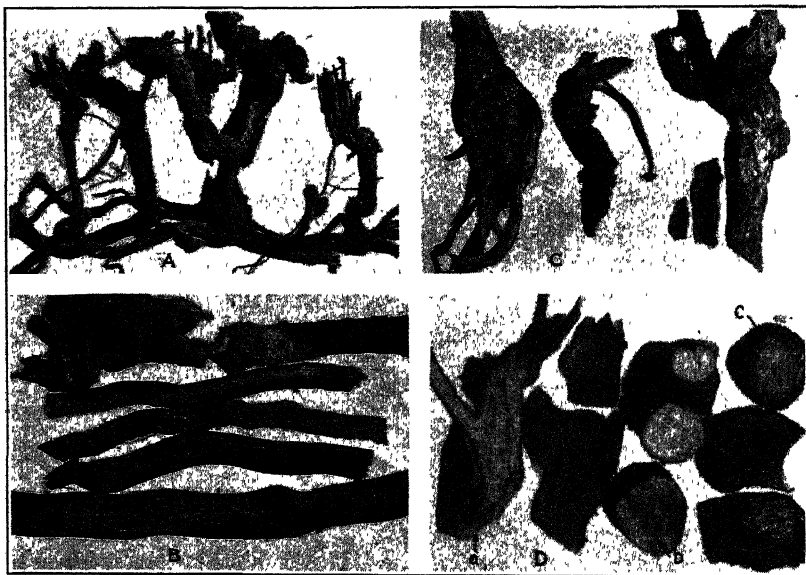


FIG. 6.—A, Underground parts of dewberry plant 10 or more years old, showing dead stumps in center and live but diseased stumps on each end. The central stumps without cane spurs have been dead for several seasons. B, Rhizomorphs and mycelium on roots and stump. C, The results of pure culture inoculation in flasks, showing characteristic web of white fungous hyphae in bark and cambium of severely infected stems. Note small undeveloped sporophore in center D, Diseased stems; showing: a, slight uninfected area supporting canes above; b, slightly discolored diseased heartwood; c, transverse section of stem and abundance of white fungous hyphae in both bark and cambium

studies new rhizomorphs and sporophores were produced for six months, after which neither rhizomorphs nor sporophores developed

in these cultures. This was obvious, since an examination of the dewberry parts used in the cultures showed that all tissues of the roots and canes were infected throughout with mycelium which had used the sustenance available to the fungus. Tests for starch and for reducing sugars gave negative results.

In cultures the mycelium is some shade of white or light tan. There are few to many strands of mycelium in a rhizomorph, and these are characterized by long, straight walls and clamp connections. In the cortex, phloem, ray tissue, and cambium the fungus produces wefts or fans of white fungous hyphae, while in the xylem and pith cells it forms single hyaline strands. The rhizomorph is a shade of white, tan, or brown, never black, being alike in pure culture and on infected parts in the soil. It is much branched and extends from one part to another in long straight strands. The largest rhizomorph obtained in these studies measured a millimeter in diameter.

By using the dewberry medium in 10 flasks, an abundance of sporophores were produced. The total production amounted to 138 mature and many immature sporophores. Twenty-eight plants were obtained in a single flask. This heavy production of sporophores has been of much assistance in the determination of the fungus. Specimens were submitted to C. H. Kauffman, of the University of Michigan, G. W. Martin, of the University of Iowa, and W. C. Coker, of the University of North Carolina, all of whom determined the fungus as a variety of *Collybia dryophila* Fr. The sporophore is very similar, and is probably the same as specimens collected by Coker and his associates in various habitats of North Carolina. Therefore, it seems advisable to add a concise but short description of the parasitic variety.

The sporophore as produced in pure culture is solitary, gregarious, and subcaespitose. (Fig. 8, A and B.) The pileus is gibbous,



FIG. 7.—A, The result of pure culture inoculation, showing breaking up of roots and stems, which is easily accomplished at the end of six months' culture, due to destructiveness of the fungus. B, Hyaline mycelium in xylem show abundance of strands in some cells

smooth, dull, subhygrophanous, white, tan, and brown, 2 to 8 centimeters broad. The lamellae are adnexed, crowded, white. The stipe is whitish changing to same color as pileus, stuffed, striate, 3 to 15 centimeters long. Spores are faint buff, pip-shaped, smooth,  $4\mu$  to  $8\mu$ .

#### INOCULATION RESULTS

The growth of a weft of fungous hyphae in bark, phloem, ray tissue, and cambium, and the mycelium in the xylem and pith cells of live wood, and persisting there throughout the year, the mass of

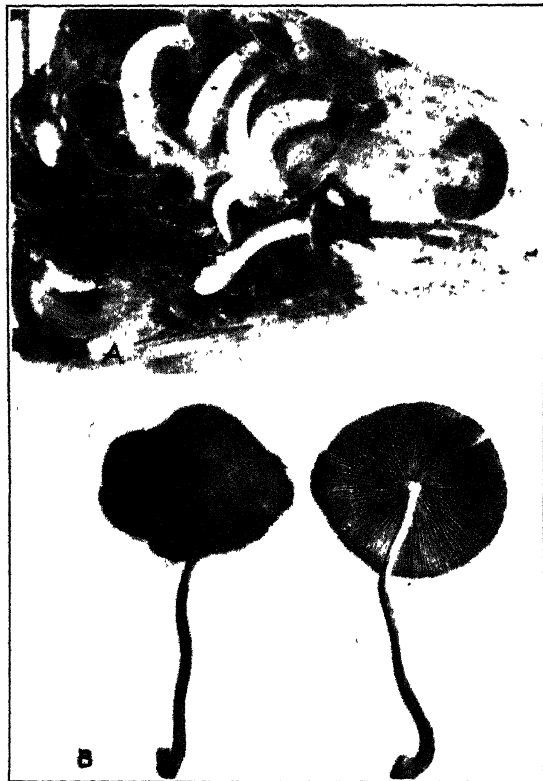


FIG. 8.—A. Pure culture development of sporophores on dewberry medium in 2-liter flask. B. Characteristic sporophore of the parasitic variety of *Collybia dryophila*

mycelium forming on and causing girdling of live epidermal cells of that part of the cane in the soil, and the conspicuous rhizomorph on live roots and stems, are prominent suspicious parasitic relations of the fungus to the plant. In order to prove its parasitism, healthy plants were inoculated with a pure culture of the parasitic variety of *Collybia dryophila*. The culture for this inoculum was 60 days old, and was grown on barnyard manure. The inoculum was placed on the roots and stems and the plants set in sand in pots in a greenhouse, where temperature and moisture conditions suitable for excellent growth of the plant and the fungus were maintained, field experiments having shown that the conditions which favored the

growth of the plant also favored the development of the fungus as a parasite. Three separate inoculation tests were conducted, each with 10 or more replications. Untreated plants were maintained under similar conditions. In all three tests the results were the same, in that 100 per cent infection was obtained on inoculated plants. The fungus produced lesions on the young roots in seven days (fig. 4, A and B), and in six months the symptoms were identical with those produced in the field (fig. 3, A). However, these studies indicate that several years are required for the complete infection and destruction by the fungus of plants grown under field conditions, but that it may kill canes in a single season. In these tests 9 per cent of the

plants were killed outright in 30 days because of badly infected roots. On the other hand 68 per cent of the infected plants have remained severely stunted while the fungus continued to attack and live on the roots nine months after the inoculum was added. (Fig. 3, A.) This behavior of the fungus under experimental conditions is similar to its behavior in nature, since the same rootstock is sometimes used for 15 years and longer, and may harbor the fungus causing continuous annual stunting of the plant without causing death. In fact the percentage of plants killed in severely infected areas is generally very small, because the deeper roots, which are not so readily destroyed, continue to produce new canes. The fungus entered the new roots without the aid of mechanical injury, but penetrated broken ends and other injured parts with equal rapidity.

In the field, infection occurs largely through the cane stubs which remain after pruning. This is easily traced on recently infected plants. The fungus enters the dead tissue, later passing into the live tissue, and as a consequence canes are killed at various periods during the growing season. (Fig. 3, B.) In this experiment the fungus girdled the small roots, which died. Later the fungus formed a felt of white hyphae on the infected parts from which the rhizomorphs extended outward into the soil. When the small infected plants were removed from the pots, mycelium and rhizomorphs were found to break off and remain in the pots. The middle lamella of cells of small roots was destroyed, and the tissues as a whole became severely infected. On the stems, the mycelium first worked into the bark, cambium, and through the rays to xylem and pith cells. (Fig. 9, A, B, C, D.) These were most conspicuously occupied by the fungus when the parts were inoculated in flasks. The fungus was isolated in April, 1927, from plants inoculated in August, 1926, and had made continuous growth on the roots and in the tissues throughout the nine months. Although apparently no gum had formed on infected tissues the adhering of soil to the plant seemed to be due partly to some excrement and partly to the matting of mycelium around the small particles. (Fig. 3, B.) Another test was conducted with live roots, which were treated for one minute in a one one-thousandth solution of mercuric chloride, washed in sterile water and added to a pure culture of the fungus in the prune medium. The same procedure was carried out with other sections, which were left uninoculated. The disease and the symptoms were readily obtained on the live roots which were killed, while the uninoculated roots remained healthy and produced new sprouts. In another test similar to this, in which partly live and dead wood was first sterilized and then inoculated, the web of hyphae became equally if not more pronounced than it is in the field. (Fig. 6, C, and fig. 9, A, B, C, D.) In all of the inoculation studies the results were more or less prominent and easily diagnosed, since the familiar mycelium, hyphae, and rhizomorphs made identification very much more certain than is possible in the study of disease symptoms produced by less conspicuous types of bacteria and fungi. Because it was possible to maintain a continuous water supply and temperature conditions satisfactory for the best growth of the fungus the definite breaking down of the tissues such as was obtained in these pure-culture studies in the laboratory, in which the wood became soft (fig. 7, A), is a

greater demonstration of the power of the fungus to destroy wood than is found on most of the severely infected plants in the field. After six months the diseased wood in the flasks is now easily torn and separated because of the action of the disease on the tissues. The untreated plants in all tests remained healthy throughout and have produced new and vigorous canes, which have been easily distinguished from the shorter and weaker growth of the diseased plants.

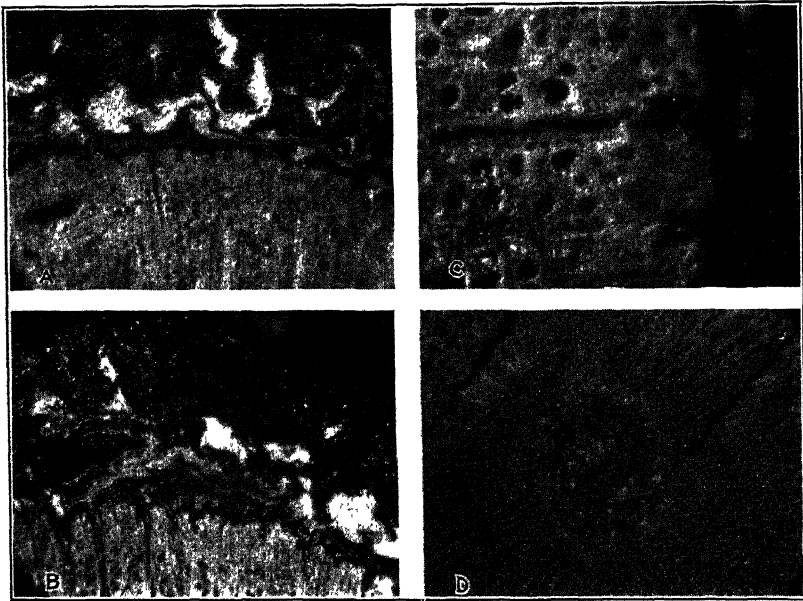


FIG. 9.—Inoculated stem of 4-year old dewberry plant, showing transverse sections made nine months after stems were inoculated with pure culture in laboratory. A, Wefts of fungous hyphae in bark and cambium. B, Fungous hyphae in the cambium, and ray cells of the growth wood. C, Enlarged ray containing strands of mycelium. D, Pith and xylem cells with hyaline mycelium.

#### DISCUSSION

This is the first report of a parasitic variety of *Collybia dryophila* Fr., a fungus which is well known and has been studied by many mycologists (1, 2, 4, 6, 7, 8). While McIlvaine and MacAdam (6) refer to the fungus as "oak-loving," they do not indicate whether or not it is a parasite on the oak.

The root-rot disease of the dewberry seems identical with the root rots of blackberry and raspberry caused by *Armillaria mellea* (Vahl) Quel. (5, 9) and to the root rot of fruit trees caused by *Clitocybe parasitica* Wilcox (10). The weft of fungous hyphae produced by all of these fungi is white and occupies the same tissues. It is obvious then that the disease caused by these fungi can not be separated solely on the basis of or the presence of mycelium and rhizomorphs—general symptoms of stump destruction and weak plant growth—when not black. While the *A. mellea* fungus has not been reported on dewberry, it attacks the blackberry (5) which is susceptible to the disease described here. It is certain that the only safe method of procedure

in determining the cause of root-rot (perhaps most appropriately stem-rot) diseases of berries, is by means of sporophores, the method by which the Agaricaceae are now classified. These fungi may live in the conductive tissues of roots and stems for many years before the production of sporophores takes place, but these can be obtained more quickly in pure culture without much difficulty, according to the present researches and to those of Gilmore (3), and Molisch (7).

The southern blackberry (*Rubus villosus* Ait.) and wild southern dewberry (*Rubus trivialis* Michx.) are both susceptible to the root-rot disease and further inoculations are being carried out to determine the relation of the fungus to other plants in the infected area.

#### SUMMARY

A root-rot disease of the *Lucretia dewberry* *Rubus procumbens* Muhl. var., *roribaccus* Bailey is causing severe damage throughout the sand hills from Sanford to Hamlet, N. C. It was reported in 1922, and appears to have become worse in recent years since plants have been plowed up in some fields because of being diseased and unprofitable, a condition which has not been described heretofore.

The symptoms of the disease are a stunted growth of the plant resulting from a diseased root system; wilting, caused by a recent attack of the disease on the canes below old spurs left after pruning, and by the death of the roots; girdling of canes; small and bushy purplish leaves; wefts of white fungous hyphae in bark, cambium, and rays, hyaline mycelium in xylem, and pith cells; white to tan and brown rhizomorphs on dead and live parts of roots and canes; and the decay of the wood, which becomes soft.

The disease is caused by a parasitic variety of *Collybia dryophila* Fr. which is widely distributed geographically in Europe and America, where it has been studied extensively, but the parasitic variety described here may not be widely distributed, since this is the first report of a parasitic variety of the *C. dryophila* "group."

The causal organism is isolated by plating on prune agar mycelium and rhizomorphs from recently infected live parts of the plant, since recently infected tissues are not apt to be infected with saprophytic fungi, which are difficult to eradicate and usually develop more quickly than the parasite. The fungus is difficult to isolate when neutral media are used, but grows well on plant coctions of potato, sweet potato, rice, apple and corn meal, on barnyard manure; and on Cook's synthetic medium, when large amounts of inoculum are transferred or when the media are made slightly acid.

The sporophores were obtained on dewberry medium only, but greater production was brought about by adding cooked prune extract to the medium. In pure culture the first sporophores were obtained in 30 days, while maximum production was obtained in from 60 to 90 days. The duration of sporophore production in this medium is six months.

The description of the parasitic variety varies only slightly from description given by others. The plant is solitary, gregarious, and subcaespitose; the pileus is white, tan, and shades of brown, and 2 to 8 centimeters broad; the stipe is striate, stuffed, and 3 to 15 centimeters long; the lamellae are some shade of white, narrow, crowded, and adnexed; the spores are hyaline, pip-shaped, and 4 to 8  $\mu$ .

The life cycle of the fungus in North Carolina is attained without uniform production of sporophores, since there were no sporophores produced in the field during 1926.

The parasitic nature of the fungus was thoroughly tested. A pure culture of the fungus was first obtained. Healthy plants were inoculated and after the disease symptoms were produced, the fungus was reisolated from the diseased areas. Furthermore, the fungus has lived on inoculated plants throughout a period of nine months, constantly forming new rhizomorphs, and attacking roots and stems.

The disease is disseminated by means of infected plants and on pruning shears.

The plants known to be susceptible to this disease are the southern blackberry (*Rubus villosus* Ait.), wild southern dewberry (*R. trivialis* Michx.), and Lucretia dewberry (*R. procumbens* Muhl. var. *roribaccus* Bailey).

The symptoms of root rots of other plants caused by *Armillaria mellea* (Vahl) Quel. and *Clitocybe parasitica* Wilcox are more or less similar to those produced by *Collybia dryophila*, from which it is obvious that identification of root-rot diseases made solely on the basis of the presence of rhizomorphs and wefts of fungous hyphae in the tissues without the sporophores, is apt to be misleading, since classification of the Agaricaceae rests upon the sporophore characters.

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# POMEGRANATE BLOTCH<sup>1</sup>

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## INTRODUCTION

The pomegranate (*Punica granatum* L.) is not grown on a commercial scale in southeastern United States, but is extensively planted as an ornamental. On this account, no doubt, little attention has been given to its diseases. During the summer of 1925, however, the writer noted the occurrence on this plant, in several localities in Florida, of a disease which affects both the foliage and the fruits. An examination showed that this disease is identical with one which was first collected in 1909 in Texas and ascribed to *Cercospora lythracearum* H. & W.<sup>2</sup> Since these brief mycological notes appear to constitute the only published account of the fungus, an attempt was made to determine its life cycle and to gain additional information in regard to the disease. It has been found that the disease not only manifests itself by foliar lesions but also by characteristic blotches upon the fruit, so that it may properly be designated by the common name "blotch." In addition it has been found that the parasite which causes blotch possesses in sequence a conidial, a spermagonial, and an ascigerous stage. The observations to date will therefore be briefly summarized as a contribution to our knowledge of pomegranate diseases.

## APPEARANCE OF LESIONS

The pomegranate in Florida retains some of its foliage throughout the entire year, so that affected leaves may be found at any season. The presence of circular or somewhat angular brown areas 4 to 5 mm. in diameter characterize the leaf-spot form of this disease. (Fig. 1, D, and pl. 1, A.) The margins of the lesions when viewed with the aid of a hand lens by transmitted light are pink and somewhat translucent. Affected leaves are pale green, and usually there are fewer than 20 spots per leaf. Diseased leaves fall prematurely in sufficient quantity to keep the ground covered. During periods of high relative humidity the lower surface of lesions is covered with dense aggregates of conidiophores and conidia which en masse appear brown. The conidial fructifications appear on the upper surface less commonly and in less abundance.

The first evidences of disease on the fruit are small but conspicuous dark-brown spots. These are circular in outline but by unequal radial growth soon become irregular blotches. By coalescence of these blotches a considerable proportion of the surface of the fruit becomes discolored. (Fig. 1, E, and pl. 1, B.) No pitting nor fissuring

<sup>1</sup> Received for publication May 20, 1927; issued October, 1927.

<sup>2</sup> HEALD, F. D., and WOLF, F. A. NEW SPECIES OF TEXAS FUNGI. *Mycologia* 3: 5-22. 1911.

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of these affected tissues occurs. When affected fruits are provided with suitable moisture conditions, such as are present when they are placed in a moist chamber, the surface of the lesions becomes covered with a velvety brown layer of conidiophores and conidia.

### THE CAUSAL FUNGUS

The *Cercospora* or conidial stage of the fungus which causes these lesions may be found on the foliage at any time throughout the year. The conidiophores occur in dense aggregates most commonly on the

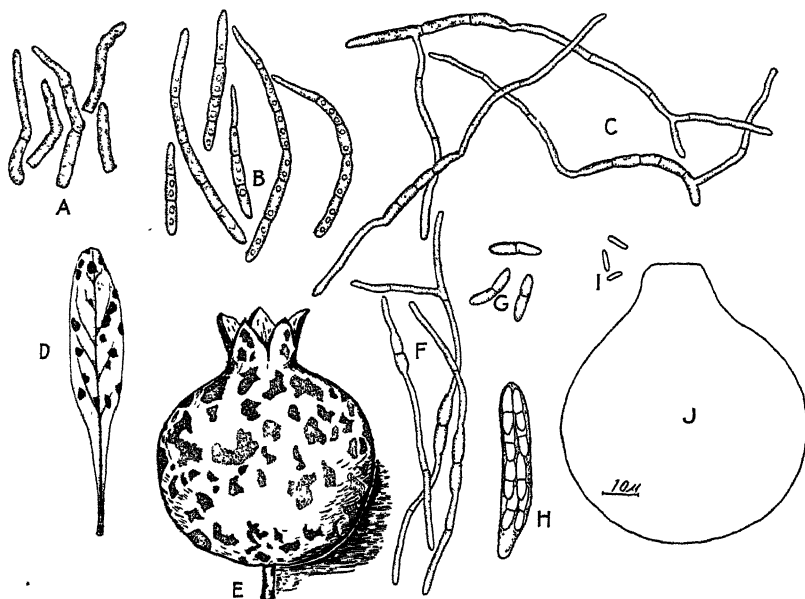
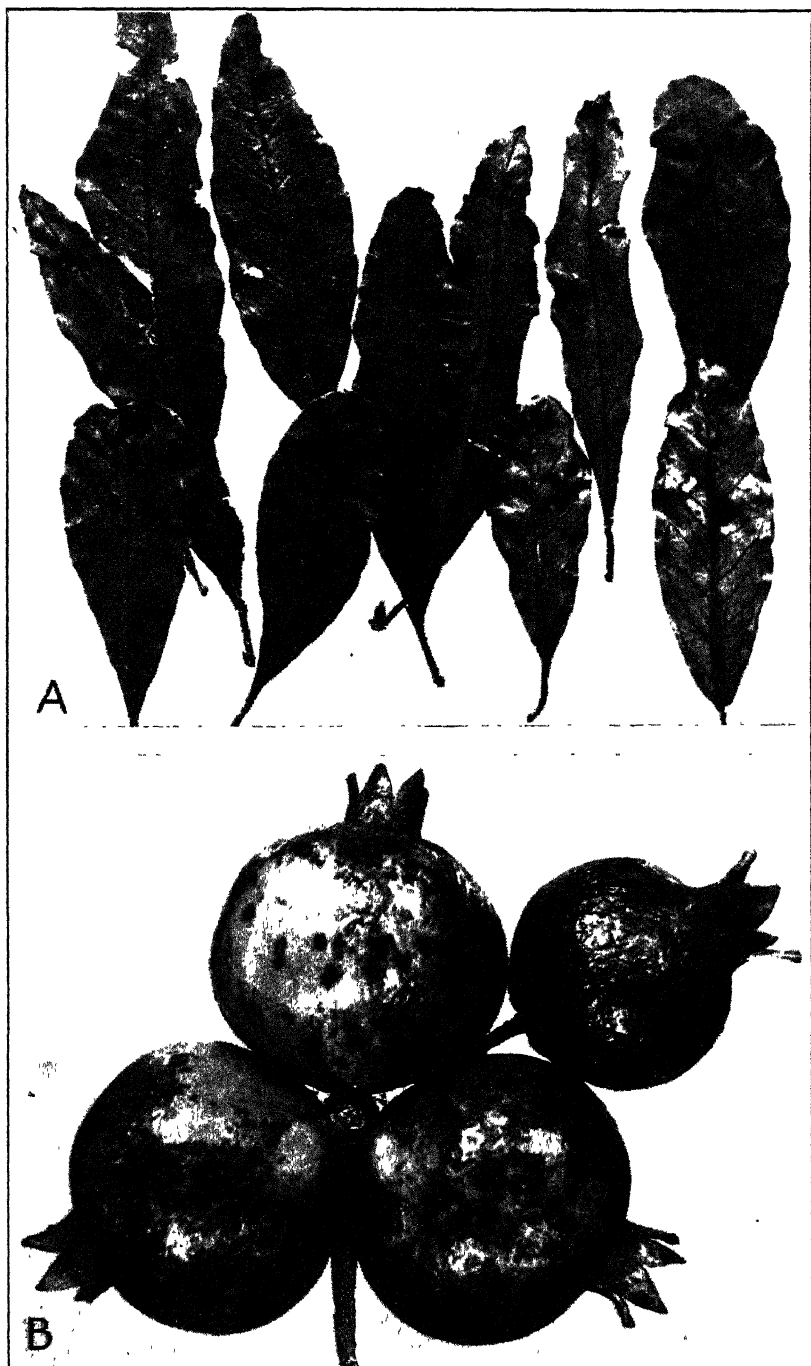


FIG. 1.—Pomegranate blotch and its causative fungus, *Mycosphaerella lythracearum*: A, conidiophores of the blotch fungus; B, conidia; C, germination of conidia; D, distribution of lesions on pomegranate leaf; E, distribution of lesions on fruit; F, germinating ascospores; G, ascospores; H, ascus; I, spermatia; J, perithecium in outline. (All except D and E drawn to scale shown in J)

lower leaf surface. They measure  $20\mu$  to  $30\mu$  by  $3\mu$  to  $4\mu$  and are faintly brown. (Fig. 1, A.) The conidia are filiform, clavate, subhyaline 2 to 5 septate, and measure  $30\mu$  to  $56\mu$  by  $3\mu$  to  $3.5\mu$ . (Fig. 1, B.) They germinate readily in water or on solid media with the formation of several germ tubes. (Fig. 1, C.) The growth of the colonies is rather slow on potato-dextrose agar, since about a month is required for the production of a colony 1 cm. in diameter. The surface of the colonies in contact with the medium is olivaceous in color and the exposed surface smoky. The mycelium is densely compacted except at the exposed surface.

Diseased leaves which were collected during August and placed in a sheltered situation in contact with the ground, when examined during October, were found to contain numerous globular black bodies in the spots which had been killed by the *Cercospora*. Free-hand sections showed that these bodies were pycnidial in structure,



A.—The leaf-spot form of pomegranate blotch  
B.—Blotch lesions on pomegranate fruits



that they opened to the lower leaf surface, and that they were filled with numerous hyaline rodlike pycnosporos measuring  $3\mu$  to  $5\mu$  by  $1\mu$  to  $1.5\mu$ . All efforts to germinate these pycnosporos have resulted in failure, which fact is regarded as evidence that they are not the conidia of a saprophytic species of *Phyllosticta* but are homologous with what have been termed spermatia. Their small size, their occurrence only in the tissues killed by *Cercospora* and not in the tissues surrounding the lesions, the time of their formation, which corresponds with that of the appearance of spermagonia of other ascomycetous fungi, and the fact that they are succeeded in due time by the perithecial stage, are regarded as additional evidence that these pycnosporos are spermatia.

At the time when the spermagonia were abundantly present, stomata were noted to be forming within the palisade parenchyma. These stomata proved from subsequent observation to be the initials of perithecia. Mature perithecia, however, were not observed until March 23. They were then present in abundance and occupied the lesions produced by the *Cercospora* stage and appeared as tiny black elevations projecting from the upper leaf surface. They are globular in outline, vary in diameter from  $75\mu$  to  $95\mu$ , and open to the surface by means of a short blunt papilla. (Fig. 1, J.) The wall is composed of a thin layer of brown cells.

The asci are eight-spored, cylindrical, without paraphyses, and cling together in a cluster when crushed out of the perithecium. They measure  $42\mu$  to  $50\mu$  by  $6.5\mu$  to  $8\mu$ . (Fig. 1, H.) The ascospores are biserial, unequally two-celled, hyaline, curved, and measure  $11\mu$  to  $14\mu$  by  $2.7\mu$  to  $3.5\mu$ . (Fig. 1, G.)

Isolations from ascospores were obtained by inverting poured potato-dextrose agar plates above leaves bearing mature perithecia. The ascospores are thus forcibly expelled and lodge on the surface of the medium. Germination occurs within 18 hours by the emergence of a germ tube from each end of the ascospore. (Fig. 1, F.) The mycelial growth is rather slow and is at first olivaceous.

Parallel cultures isolated from conidia and from ascospores were made on carrot agar, Czapek's agar, Dox's agar, prune agar, glycerine agar, cornmeal agar, potato-dextrose agar, apple agar, peach agar, sterilized pigeon-pea stems, potato cylinders, and sterilized leaves, twigs, and fruits of pomegranate. All cultures on the same medium were entirely similar in growth characters. Further, all cultures both from ascospores and from conidia have remained sterile, so that it has been impossible to secure evidence of the genetic relationship of conidial and ascogenous stages in the usual manner. This failure to form conidia in culture has precluded the possibility of making inoculations from pure cultures as a means of showing the relationship of the two stages and at the same time of proving the pathogenicity of this fungus. However, there is ample reason to believe that the two stages are related, because (1) the colonies from conidia and from ascospores are indistinguishable; (2) conidia, spermagonia, and the perithecial initials are present at the same time in autumn in the same lesions; (3) the perithecia are present in abundance in spring in the areas resultant from infection by the *Cercospora* stage; (4) they do not occur in the tissues surrounding the lesions produced by *Cercospora*, and no other ascomycete is associated with the diseased areas; and (5) these observations accord with well-known facts, since

other species of *Cercospora* are known to possess an ascigerous stage of the type described for the pomegranate fungus. Further, field observations leave no doubt that the *Cercospora* stage is virulently parasitic.

### SYSTEMATIC DESCRIPTION

The characters presented by this fungus are manifestly those of the genus *Mycosphaerella* as employed by Johanson,<sup>3</sup> to which genus it is assigned. Apparently no ascomycete of this genus has been described on *Punica granatum*, and it is not evidently like any of the *Mycosphaerellas* on *Lythraceae* or closely related plants. Furthermore, it is not believed to be advisable solely on the basis of morphological similarity to assign it to any of the numerous species of *Mycosphaerella* which have been described on other hosts. Since the *Mycosphaerella* under consideration is associated with pomegranate, and since it is highly probable that it is connected with *Cercospora lythracearum*, it may appropriately be given the name *Mycosphaerella lythracearum* with the following diagnosis:

***Mycosphaerella lythracearum* n. sp.<sup>4</sup>** Syn. *Cercospora lythracearum* Heald and Wolf.

*Ascigerous stage*.—Perithecia black, epiphyllous, aggregated on spots killed by conidial stage, globose,  $75\mu$  to  $95\mu$  in diameter, immersed with papilla protruding through the epidermis. Asci cylindrical, clustered, paraphysate,  $42\mu$  to  $50\mu$  by  $6.5\mu$  to  $8\mu$ ; spores biserial, slightly curved, colorless, unequally once septate,  $11\mu$  to  $14\mu$  by  $2.7\mu$  to  $3.5\mu$ . In fallen leaves of *Punica granatum*.

*Conidial stage*.—Lesions on leaves angular to circular, brown, 4 to 5 mm. in diameter; on fruit, irregular dark blotches. Dense aggregates of conidiophores  $20\mu$  to  $30\mu$  by  $3\mu$ , subhyaline, amphigenous, mostly hypophyllous. Conidia filiform, clavate,  $30\mu$  to  $56\mu$  by  $3\mu$  to  $3.5\mu$ , hyaline, 2 to 5 septate. Parasitic on leaves and fruits of *Punica granatum*.

*Spermagonial stage*.—In autumn epiphyllous spermagonia appear in lesions produced by the *Cercospora* stage. Spermata  $3\mu$  to  $5\mu$  by  $1\mu$  to  $1.5\mu$ .

Available records of collections indicate the occurrence of pomegranate blotch in several localities in Texas, Florida, Alabama, and Bermuda.<sup>5</sup>

### LATIN DESCRIPTION

Peritheciis atris, epiphyllis, gregariis, globosis, epidermide velatis poro perituis,  $75-95\mu$ ; ascis cylindricis, fasciculatis, paraphysatis,  $42\mu$  to  $50\mu$  by  $6.5\mu$  to  $8\mu$ ; sporidiis distichis, leviter curvulis, hyalinis, bicellularibus, loculis subaequalibus,  $11\mu$  to  $14\mu$  by  $2.7\mu$  to  $3.5\mu$ . Hab. in foliis dejectis *Punicae granati*. Status conidicus: maculis in foliis angularis vel rotundatis, brunneis, 4 to 5 mm. in diam.; laesiones in fructis irregularibus, atris; conidiophoris dense aggregatis,  $20\mu$  to  $30\mu$  by  $3\mu$ , dilute brunneis; conidiis filiformibus, clavatis,  $30\mu$  to  $56\mu$  by  $3\mu$  to  $3.5\mu$ , hyalinis, 2 to 5 septatis; spermatis autumnis in spermagoniis,  $3\mu$  to  $5\mu$  by  $1\mu$  to  $1.5\mu$  hypophyllis. Hab. in foliis fructisque vivis *Punicae granati*.

Specimens have been made available by deposition in the collections of the Office of Mycology and Disease Survey, Bureau of Plant Industry, Washington, D. C.

### SUMMARY

This report concerns a disease which appears on the foliage and fruits of *Punica granatum*. It has hitherto been known as a leaf spot but manifests itself also by characteristic blotches on the fruit. It is known to occur in Texas, Florida, Alabama, and Bermuda.

<sup>3</sup> JOHANSON, C. J. SVAMPAR FRÅN ISLAND. Öfvers. K. [Svenska] Vetensk. Akad. Förhandl. 41 (9): 163, 1894.

<sup>4</sup> There are mycologists who would use the generic name *Sphaerella* in the sense employed in the following: SACCARDO, P. A. SVILLOGE FUNGORUM. v. 22, p. 120. Patavii. 1913.

<sup>5</sup> Collections from Alabama were made by H. R. Fulton and from Bermuda by H. H. Whetzel and L. Ogilvie.

Pomegranate blotch is caused by an ascomycetous fungus herein given the name *Mycosphaerella lythracearum* n. sp. The conidial stage of this fungus, *Cercospora lythracearum*, is present throughout the entire year in Florida on green foliage.

A spermagonial stage occurs during autumn on the lower surface of lesions caused by the *Cercospora* stage.

Mature perithecia are present in early spring on fallen leaves.

The relationship of conidial and perithecial stages is evidenced (1) by their occurrence in due time in the same lesions, (2) by the similarity of cultures from conidia and ascospores, and (3) by the fact that a number of *Cercosporas* are known to possess a *Mycosphaerella* stage.



# A SUBCUTANEOUS TUMOR IN A PRIMATE CAUSED BY TAPEWORM LARVAE EXPERIMENTALLY REARED TO MATURITY IN DOGS<sup>1</sup>

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Agriculture

## INTRODUCTION

This paper contains a description and discussion of a tapeworm of which the larval stage, known to zoologists as a coenurus, was found causing a large subcutaneous tumor in a primate (*Theropithecus obscurus*), and from which the adult stage, a strobilate tapeworm, was experimentally reared in dogs. Despite the fact that these bladder worms and experimentally reared tapeworms as investigated by the writer appear to be morphologically indistinguishable from one of the known dog tapeworms (*Multiceps serialis*), the former are regarded in this paper as representing a variety of that species. The reasons for differentiating these worms from *Multiceps serialis* are based on experiments involving the intermediate host relationship of the parasite studied by the writer. The latter form will be referred to in this paper as *Multiceps serialis* var. *theropithecii*. This designation conforms to a certain tendency among parasitologists to designate varieties of parasites by the generic name of the host or by a derivative of that name.

Parasites of primates have considerable potential significance in human medicine, as Stiles and Orleman (10)<sup>2</sup> have recently noted, and this fact warrants a detailed study of this coenurus as possibly one that is capable of developing in man with distinctly unpleasant consequences. As a matter of fact two very closely related larval tapeworms, *Multiceps multiceps* and *Multiceps glomeratus*, have been reported from man, the former from one of the lateral ventricles of the brain and the latter from one of the intercostal muscles.

The parasites were collected by Robert Formad, of the pathological division of the Bureau of Animal Industry, in the course of a necropsy on a baboon (*Theropithecus obscurus*) from the National Zoological Park at Washington, D. C. The baboon was killed early in April, 1925, because it was deformed by a large subcutaneous tumor in the right thoracic region which rendered the animal unsuitable for exhibition purposes. Formad removed the tumor, recognized it as parasitic in nature, and referred it to the zoological division for identification. The following is a part of a report submitted by Formad in regard to the post-mortem appearance of the tumor:

Carcass in good condition. Large cystic tumor, parasitic in nature and honey-combed in appearance, was situated externally on the right thoracic region, adhering to the skin around the nipples. A fistulous and necrotic tract extended for about 1 inch into the tumor.

<sup>1</sup> Received for publication Apr. 26, 1927; issued October, 1927.

<sup>2</sup> Reference is made by number (italic) to "Literature cited" p. 480.

## DESCRIPTION OF TUMOR

In its unpreserved state the tumor weighed about 1,500 gm. The preserved specimen is more or less spherical in shape and somewhat asymmetrical, being about 20 cm. long by about 16 cm. wide. Its membranous surface presents a number of openings of which some are more or less circular, others irregular in shape. The openings lead into spaces which communicate with adjoining spaces through openings, the entire tumor presenting a honeycombed appearance and the intercommunicating spaces being more or less completely filled with bladder worms (larval tapeworms).

About one-half of the membranous surface of the cyst appears wrinkled and folded, with numerous bladder worms protruding through the openings. The remaining portion of the external surface of the cyst is fairly smooth and contains but few openings. The general shape and appearance of the tumor are shown in Plate 1, in which a number of the larger openings on the wrinkled surface of the cyst wall are clearly visible.

## DESCRIPTION OF BLADDER WORMS

Many of the bladder worms are more or less elliptical, some are spherical, and others are irregular in shape. They occur in clusters,



FIG. 1.—A cluster of bladder worms showing the attachment of the coenuri to the cluster by means of stems. About natural size

the individuals containing, as a rule, several heads. The bladder worms show considerable variation in size and shape and in external modification, most of them containing a variable number of fringed structures on the surface. Some appear to be perfectly smooth, however, and occasionally occur as single forms with a small stalk or pedicle by which they were probably at one time attached to a cluster. Although the bladder worms are commonly found in grape-like clusters, some forms exhibit a branching structure, being attached to the parent stem by short stalks. The appearance of the colonial bladder worms and their union to one another as well as their mode of branching are shown in Plate 2 and in Figure 1, in which various shapes of individual bladder worms, cluster formation, and the attachment of bladder worms to stalks by means of short stems are clearly illustrated.

Although a number of sterile bladder worms were observed, as well as a number which contained but a single head each, the majority of the mature individuals contained several heads each. Figure 2 shows a cluster containing two large and several smaller bladder worms. The large bladder worms contain several heads each, most of the heads being evaginated. Figure 3, *a*, shows a bladder worm with eight invaginated and one evaginated head, and Figure 3, *b*, shows a bladder worm with seven invaginated heads and a smaller bladder worm attached to it with three invaginated heads.

The question of the specific identity of the coenurus stage of the tapeworm genus *Multiceps* involves primarily the number, size, and shape of the hooks and incidentally the other head structures, notably the suckers and rostellum. Examination of the bladder-worm heads obtained from the tumor showed them to be densely



Tumor showing openings on membranous surface. The irregularly shaped, whitish objects are the protruding bladder worms



A group of bladder worms, *Multiceps serialis* var. *theropithecii*. Natural size. *a*, A small group of bladder worms, the one on the right being attached by means of a small stem; *b*, *c*, and *d*, groups of bladder worms showing variation in size and shape of the individual coenuri; *e*, a grapelike cluster of bladder worms; *f*, a group of bladder worms that exhibit a dendritic mode of branching; *g*, two clusters of bladder worms connected by a strand of tissue

crowded with calcareous corpuscles. In unpressed preparations the diameter of the scolex or head is about  $774\mu$ ; the diameter of the suckers ranges from  $235\mu$  to  $260\mu$ . There is a double crown of from 28 to 32 hooks. In one specimen the large hooks range in length from  $135\mu$  to  $144\mu$  and the small hooks from  $90\mu$  to  $95\mu$ . In another specimen the following measurements of the individual large hooks were made:  $144\mu$ ,  $135\mu$ ,  $135\mu$ ,  $139\mu$ ; and in the same specimen the following measurements of the lengths of the small hooks were made:  $95\mu$ ,  $90\mu$ ,  $81\mu$ ,  $95\mu$ ,  $90\mu$ ,  $95\mu$ . In still another specimen four large hooks were  $135\mu$  long, one was  $138\mu$ , another  $140\mu$ , another  $144\mu$ , and one  $148\mu$ . Small hooks in the same specimen showed the following lengths: Two,  $90\mu$ ; three,  $95\mu$ ; two,  $99\mu$ ; one,  $108\mu$ ; and one,  $118\mu$ . The extremes are  $135\mu$  to  $148\mu$  for large hooks and  $81\mu$  to  $118\mu$  for small hooks.

The large hooks (fig. 4) have a blade of moderate curvature, a short thick handle, sinuous in outline and distinctly marked off at the point of union with the blade by a curvature, the convexity of the curve being ventral. The guard is short, thick, and bluntly rounded. The small hooks (fig. 4) have a blade of from moderate to strong curvature, a short, thick, blunt handle, and a guard resembling that of the large hooks.



FIG. 2.—A cluster consisting of two large and several smaller bladder worms, most of the heads being evaginated. Enlarged

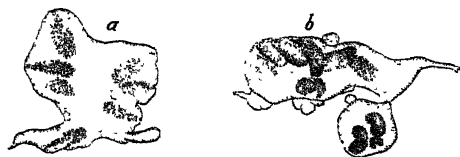


FIG. 3.—Bladder worms with evaginated heads. Enlarged

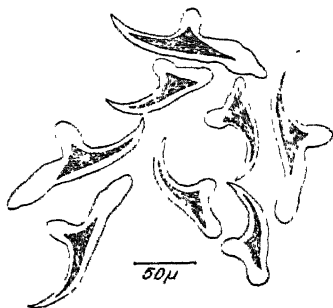


FIG. 4.—Large and small hooks of larval worms, *Multiceps serialis* var. *theropithecii*

#### KNOWN SPECIES OF MULTICEPS

In recent years Railliet and Mouquet (7) and Joyeux (3) have summarized the known species of *Multiceps*, a total of 12 species being referred to that genus as follows: (1) *M. multiceps* (Leske, 1780), the intermediate (coenurus) stage of which occurs in the brain and spinal cord of sheep and other herbivores and the adult stage in dogs. Records of the occurrence of the larval stage of this tapeworm

in a hog and in man have also been published. (2) *M. serialis* (P. Gervais, 1847), the larval form occurring in subcutaneous and in intermuscular tissue of various Leporidae and other rodents, the adult stage in dogs. (3) *M. lemuris* (Cobbold, 1861), the larval form occurring in the liver, lungs, and pleura of *Lemur catta*, the adult stage being unknown. The host of this parasite has been referred to as *Lemur macaco*. Railliet and Mouquet (?) have shown that the specific name of the host is *L. catta*. (4) *M. polytuberculosus* (Méglin, 1879), the larval form occurring in a rodent (*Jaculus jaculus*) according to Railliet and Mouquet (?). (5) *M. brauni* (Setti, 1897), the adult stage occurring in the dog, and the larval form occurring under the skin of a mouse (*Gerbillus pyramidum*), according to von Linstow (5). (6) *M. glomeratus* Railliet and Henry, 1915, the larval form occurring in the abdominal cavity of a mouse (*Gerbillus hirtipes*), the adult stage being unknown. (7) *M. spalacis* (Diesing, 1863), the larval form occurring in *Georhynchus capensis*, the adult form being unknown. (8) *M. gaigeri* Hall, 1916, the larval form occurring under the skin, in the intermuscular tissue on the viscera, and in the central nervous system of the goat (*Capra hircus*), the adult stage occurring in the dog. (9) *M. ramosus* Railliet and Marullaz, 1919, the larval form occurring in subcutaneous tissue of the perineum of *Cynomolgus (Macaca) sinicus*, the adult stage being unknown. (10) A somewhat doubtful form, *M. (?) ovis tragelaphi* (Cobbold, 1861), the larval form occurring in *Ovis tragelaphus* and, according to Meggitt (6), in *Ammotragus lervia*, the adult being unknown. (11) *M. clavifer* Railliet and Mouquet, 1919, the larval form occurring in a rodent (*Myopotamus coypus*), the adult stage being unknown. To the above species must be added (12) *Multiceps radicans* Joyeux, Richet, and Schulmann 1922 (4), the larval form occurring in a white mouse, the adult stage being unknown.

As regards the size of the hooks, the coenurus from the baboon which is described in this paper corresponds to *Coenurus (Multiceps) serialis*. The only other species to which it may possibly correspond on the basis of the size and number of hooks is *Multiceps brauni*. The latter species has large hooks ranging from  $130\mu$  to  $140\mu$  long, occasionally only  $95\mu$  to  $100\mu$  long, and small hooks ranging from  $85\mu$  to  $90\mu$ , occasionally only  $70\mu$  to  $75\mu$  long, these measurements being based on the adult stage. Both the large and the small hooks of the specimen from the baboon exceed these measurements. According to Hall (2), the guard of the large and small hooks of *M. brauni* tends to be bifid, a character not observed in the present writer's specimens from the baboon. So far as concerns the morphology of the hooks, which constitutes the best character on which to base a determination of a larval taenioid tapeworm, the specimens from *Theropithecus obscurus* appears to agree with those of *Multiceps serialis*.

#### EXPERIMENTAL TRANSMISSION OF THE COENURUS FROM THEROPITHECUS TO DOGS

In order to determine the adult stage of the tapeworm in question, feeding experiments were performed and the results noted in each case. These results, briefly summarized in a previous paper (8), follow:

## EXPERIMENT 1

On April 8, 1925, dog 868 was fed one large cyst containing several heads. A fecal examination of this dog prior to feeding the bladder worm yielded the following results: Ova of *Toxascaris*, *Dipylidium*, and *Trichuris* present. The dog died April 29, 21 days after the cyst had been fed. The following parasites were removed from the dog at necropsy: *Toxascaris limbata*, *Trichuris vulpis*, *Dipylidium caninum*, and four small taenioid tapeworms (*Multiceps*). The total length of each tapeworm before fixation was ascertained to be 9.2 cm., 7.7 cm., 4.4 cm., and 2.3 cm., respectively. The head characters of these tapeworms were identical with those of the larval forms which were fed to the dog. The following are the detailed measurements of one specimen: Total length after fixation, 7.2 cm.; total number of hooks, 32; diameter of head,  $732\mu$ ; diameter of rostellum,  $270\mu$ ; large hooks,  $144\mu$  to  $149\mu$  long; small hooks,  $90\mu$  to  $95\mu$  long. A short distance below the neck the segments are  $72\mu$  long by  $666\mu$  wide; from this point the segments gradually increase in length to  $144\mu$  without any appreciable increase in width. Segments  $306\mu$  to  $324\mu$  long are about  $864\mu$  wide; segments  $918\mu$  long are 1.2 mm. wide and show the presence of testes.

## EXPERIMENT 2

Dog 890 was fed several bladder worms on April 8, 1925, containing a total of about 15 heads. This dog was not examined for parasites before feeding, but several days after feeding an examination was made and the following parasite ova were found: *Toxascaris*, *Dipylidium*, and *Taenia*. The dog was killed on June 6, 1925, approximately two months after experimental feeding. In addition to *Toxascaris limbata*, *Dipylidium caninum*, and *Taenia pisiformis*, the dog contained about a dozen specimens of *Multiceps*. Two specimens of the last-named species were examined and the total number of hooks found were 30 and 32, respectively. The large hooks were  $145\mu$  to  $153\mu$  long; the small hooks from  $90\mu$  to  $95\mu$ . The suckers are from  $235\mu$  to  $252\mu$  in diameter.

## EXPERIMENT 3

Dog 918 was fed several bladder worms on April 11, 1925. Fecal examination of this dog prior to feeding showed the presence of numerous *Belascaris* ova. On May 25, approximately six weeks after experimental feeding, several tapeworm proglottids and one head were found in the feces of this dog. The hooks corresponded in size and number to those of the bladder worms fed. On June 1, 1925, about seven weeks after experimental feeding, proglottids containing eggs were found in the feces of this dog. Two days later additional proglottids containing eggs were found in the feces. This dog died on June 14, 1925, but was accidentally disposed of without being examined.

## EXPERIMENT 4

Dog 919 was fed several bladder worms on April 11, 1925. A fecal examination two days later showed the presence of *Ancylostoma* and *Trichuris* ova. On May 8, 1925, approximately four weeks after feeding, the dog was killed. Post-mortem examination revealed the presence of the following parasites: *Trichuris vulpis*, *Dipylidium caninum*, *Anchylostoma caninum*, and 15 taenioid tapeworms (*Multiceps*).

ceps) with heads. One of the tapeworms was found in the duodenum, the remaining specimens being found in the jejunum and ileum, the latter portion of the intestine containing fewer tapeworms than the former. Several of the tapeworm heads from this dog were examined and they corresponded morphologically to those recovered in the previous feeding experiments described above.

#### EXPERIMENT 5

On April 7, 1925, several bladder worms each containing two or more heads were fed to a cat. This animal was kept under observation for more than two months during which period its feces was examined from time to time for proglottids and for ova but with negative results.

#### MORPHOLOGY OF TAPEWORMS EXPERIMENTALLY REARED IN DOGS AS A RESULT OF FEEDING THEM COENURI FROM THEROPITHECUS

The head of the tapeworm is more or less quadrangular when viewed en face (fig. 5), its diameter varying from about  $738\mu$  to slightly over 1 mm. The rostellum

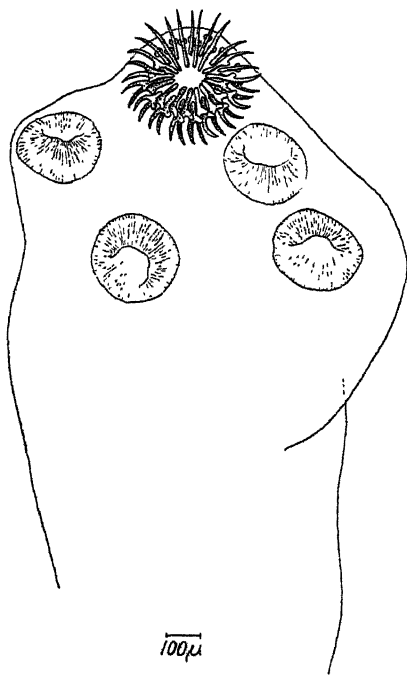


FIG. 5.—Head of strobilate tapeworm, *Multiceps serialis* var. *theropitheci*

is from  $270\mu$  to about  $360\mu$  in diameter, bearing a double crown of from 28 to 32 hooks. The large hooks (fig. 6, A and D) are from  $144\mu$  to  $153\mu$  long. The blade is of moderate curvature; the handle is somewhat sinuous in outline, rather short, and of varying shape and diameter. In some of the large hooks the handle is slightly tapering, in others it ends bluntly. The handle of practically all the large hooks that were examined showed a tendency to turn dorsally at the distal extremity. The guard is short, its distal extremity being blunt in some specimens and somewhat tapering in others. The small hooks (fig. 6, B and C) are from  $90\mu$  to  $103\mu$  long. The blade is of moderate curvature. The handle is short and thick. In some specimens the handle shows a slight curvature, the convexity of the curve being ventral; in other specimens this curvature appears to be absent. The guard is short and

thick, its distal extremity being more or less pointed. The suckers are about  $270\mu$  in diameter. The entire strobila is from 29 to 48 cm. long. The neck is very distinct. The mature segments (figs. 7 and 8) are wider than long, about 2 mm. long by about 3 mm. wide. The gravid segments are longer than wide, usually 7 or 8 mm. long, occasionally about 10 mm. long by 3 to 4 mm. wide. The entire strobila

is very dense and opaque. The genital pores are irregularly alternate. The genital papilla is very prominent, being situated in the middle of, or, more usually, somewhat posterior to the middle of the segment.

#### MALE GENITALIA

In immature specimens the testes do not extend to the field of the median stem of the uterus, but in fully mature specimens the testes spread to this field (figs. 7 and 8) and extend close to the margin of the vagina and the vas deferens. The testes also extend to the margin of the ovaries and posteriorly beyond the ovaries to the vitellarium, but not behind the vitellarium. The vas deferens originates at some distance from the median uterine stem on the pore side of the proglottid. The cirrus pouch is transversely elongated and narrow.

#### FEMALE GENITALIA

In some segments the ovaries are only slightly dissimilar in size; in other specimens there is a marked difference in size between the two ovaries. The vitellarium is elongated along the transverse axis of the segment. In some specimens it does not extend laterally as far as the margins of the ovary; in other specimens it reaches the margins. In some segments (fig. 8) the vagina

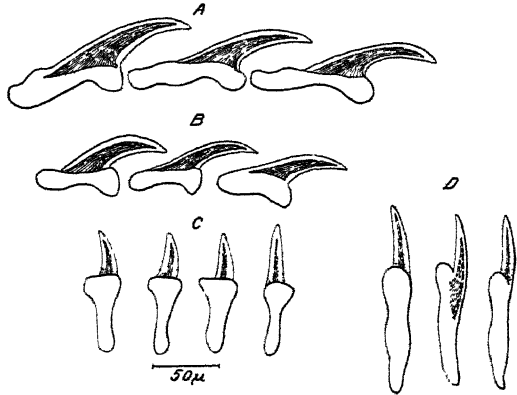


FIG. 6.—Large and small hooks of strobilate tapeworm, *Multiceps serialis* var. *theropithecii*. Enlarged

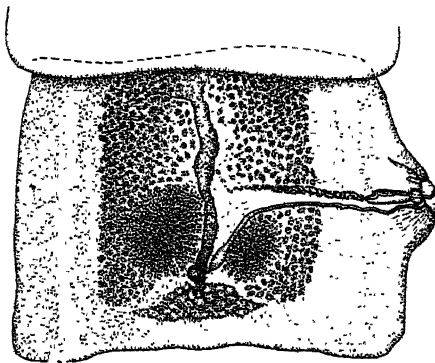


FIG. 7.—Sexually mature segment of *Multiceps serialis* var. *theropithecii*. Enlarged

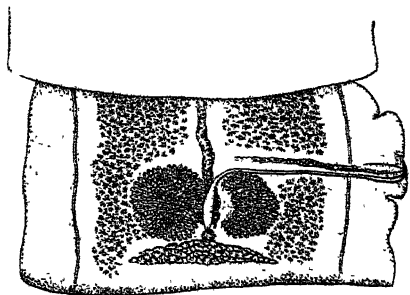


FIG. 8.—Sexually mature segment of *Multiceps serialis* var. *theropithecii*. Enlarged

shows no loop and is practically straight; in other segments (fig. 7) it is somewhat sinuous in the region of the excretory canal, and a more marked loop than that shown in Figure 7 was observed in a number of segments. The gravid segments show a median uterine stem with numerous branches and subbranches, making an actual count difficult. The eggs are variable in size, ranging from  $25\mu$  to  $42\mu$  in diameter.

The morphology of the adult tapeworm corresponds closely to Hall's (2) description of *Multiceps serialis*. From the description of *M. serialis* as given by Hall and from an actual study of specimens of that species, no constant characters have been found that would serve to differentiate this worm from the form from the baboon, which was experimentally reared in four dogs.

#### EXPERIMENTS ON THE ATTEMPTED TRANSMISSION OF THE HEXACANTH EMBRYOS TO VARIOUS EXPERIMENTAL ANIMALS

In order to obtain additional evidence that would throw light on the specific identity of this tapeworm, the following experiments were performed:

##### EXPERIMENT 6

Two gravid proglottids from dog 918 were fed to each of two rabbits on June 1, 1925. One rabbit died on July 6, 1925. Post-mortem examination showed no evidence of infestation with larval tapeworms. The second rabbit was killed a month later, and the results were likewise negative.

##### EXPERIMENT 7

On June 4, 1925, a gravid segment from dog 918 was fed to a guinea pig which was killed within two months. The results were negative.

##### EXPERIMENT 8

A ripe proglottid from dog 918 was chopped up and fed on bread to three white mice. The mice were killed six weeks later and showed no evidence of infestation with larval tapeworms.

##### EXPERIMENT 9

On June 13, 1925, one gravid segment from dog 918 was fed to each of two guinea pigs and one gravid segment from the same dog was fed to one rabbit. The guinea pigs were killed two months later and the rabbit six months later. The results were negative.

##### EXPERIMENT 10

On June 24, 1925, each of two rabbits was fed one gravid segment from dog 890 and each of three guinea pigs was fed one-half gravid segment from the same dog. Chopped up gravid proglottids from this dog were also fed on bread on the same day to six rats. These animals were kept for about six months. Post-mortem examinations were negative.

##### EXPERIMENT 11

Two gravid segments from dog 890 were fed to each of two lambs on June 23, 1925. One lamb was accidentally disposed of so that no post-mortem examination was made. The second lamb was killed about five months after feeding and the results were negative.

#### DISCUSSION

The failure to rear the larval stage of this tapeworm in rabbits throws some doubt on the biological identity of the worm with *Multiceps serialis*, since the rabbit is the normal intermediate host of this species of tapeworm. While the worm from *Theropithecus* is morphologically related to, if not identical with, *M. serialis* it

appears to be biologically distinct, as far as can be judged by the results of feeding experiments described in this paper. While admitting the soundness of the proposition that zoological species should be based on morphological characters, it is important in the case of parasites to take cognizance of biological differences as evidenced by host relationship, and on the ground of an apparent biological distinction between *M. serialis* and the form discussed in this paper, the writer is inclined to regard the species from *Theropithecus* experimentally reared in dogs as a variety of *M. serialis*, for which the name *Multiceps serialis* var. *theropithecii* is proposed. Assuming that the two forms are morphologically identical, it may be suggested as an interesting speculation that in adapting themselves to new and unusual hosts parasites may undergo profound changes in their physiological organization without any apparent corresponding morphological modifications, and that such physiological species probably constitute the starting point which may ultimately lead to a definite morphological deviation from the parent species. An apparent physiological differentiation appears to have occurred in genera of parasitic mites such as *Sarcoptes*, *Psoroptes*, *Chorioptes*, and *Notoedres*, as some species of these genera from different hosts appear to be morphologically identical but are apparently more or less restricted to their host species. Among nematodes the case of *Ascaris lumbricoides* is of special interest in this connection. Although the human and swine *Ascaris* appear to be morphologically indistinguishable, considerable experimental and epidemiological evidence has been accumulated in recent years which shows that the form from the human host does not reach fertile maturity in the pig and vice versa. A similar case is that of the hookworm *Necator americanus* which occurs in human beings with a very similar form occurring in the pig, those from the latter host having been described as *N. suillus*. The two forms are very closely related and morphologically very similar. Attempts on the part of various workers to transmit experimentally *N. americanus* of human origin to pigs have invariably yielded negative results.

A similar host parasite relationship appears to hold true in certain plant parasitic nematodes, and this subject has been recently summarized by Steiner (9) as follows:

A plant-parasitic nema apparently prefers always the host species or even the host variety on which its parents lived; the host principle, as established by Hopkins for insects, is also applicable to our plant-parasitic nemas.

This preference for a certain host grows with the number of generations a nema population lives on it, the latter getting more and more specialized on this very host. This specialization may reach such a high degree that finally even new hosts of the closest taxonomical, physiological and chemical relationship to the old host are attacked no more or very lightly.

In the case of the species discussed in this paper, *Multiceps serialis* var. *theropithecii*, the host relationship is one that involves the larval stage only, the adult host being the same as that of *M. serialis*. The apparent intermediate host specificity of *M. serialis* var. *theropithecii* is correlated in this particular case with a larger cyst which has a more complex mode of branching than has been previously observed for *M. serialis*.

## SUMMARY

A large subcutaneous cyst from a baboon (*Theropithecus obscurus*) is described in this paper as due to larval cestodes belonging to the genus *Multiceps*. A comparison of the hooks and of other morphological characters of this species of *Multiceps* with the other known species of the genus shows that the form from *Theropithecus* is closely related to, if not identical with, *Multiceps serialis*.

As a result of feeding one or more bladder worms (coenuri) to each of four dogs, the adult tapeworm was experimentally reared in these animals and its morphological organization was found to be similar to, if not identical with, that of *Multiceps serialis*. Owing, however, to a failure to develop the larval stage of the tapeworm in rabbits as a result of feeding them gravid proglottids of the tapeworms experimentally reared in dogs, the specific identity of the worm is open to question and in order to avoid confusion it is given varietal rank, the name *Multiceps serialis* var. *theropithecii* being proposed for it.

It is suggested that *Multiceps serialis* var. *theropithecii* probably represents a physiological species that has become adapted to a new intermediate host. The possibility that such host adaptations may ultimately lead to species differentiation among parasites appears to be indicated as a possibility.

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## STUDIES OF THE PHYSIOLOGICAL ANATOMY OF THE STRAWBERRY<sup>1</sup>

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### INTRODUCTION

The material herewith presented is the result of a study of the physiological anatomy of the strawberry (*Fragaria* spp.) undertaken in connection with the work in breeding this genus which is being carried on by George M. Darrow, of the Bureau of Plant Industry. The present paper is based on the anatomy of the varieties known as "Howard 17" and "Progressive" (both probably descended from crosses between *Fragaria chiloensis* and *F. virginiana*), supplemented as regards certain features particularly pertinent to the problems in mind, by comparison with eight other characteristic species and varieties.

Some of the specific questions which have presented themselves for consideration are: (1) The runner being such a slender, flexible, exposed organ, what are the structural features which enable it to carry sufficient water and nutrients for the maintenance of many runner plants with their large leaf areas before these have established direct contact with the soil? (2) How long is a runner morphologically; is it composed of one, two, or several internodes? (3) Why does the first runner from a runner plant develop so much more rapidly than do the later ones? (4) Why are plants having only the large, white roots, so commonly observed, less likely to succeed on transplantation than those without such roots? (5) What is the structure which enables water and nutrients to move freely from one side of the plant to the other?

Except for the incidental references contained in the general works of Gérard (2),<sup>3</sup> Reinke (4), Trécul (7), Solereder (5), and Van Tieghem and Douliot (8), no information concerning the anatomy of the strawberry is available, as far as could be learned.

### MATERIALS AND METHODS

The plants used in these studies were obtained from the breeding plots of the Bureau of Plant Industry at the Bell Horticultural Field Station at Glenn Dale, Md., and the preparations were made in the botanical laboratories of Johns Hopkins University at Baltimore. Most of the sections and other preparations were made from plants collected in early November and fixed in medium chromacetic fixative. These were supplemented with fresh materials for dissection and for

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<sup>2</sup> Acknowledgment is given to those who have aided in this work, particularly to George M. Darrow for the original problem and to D. S. Johnson, professor of botany in Johns Hopkins University, for the freedom of the Johns Hopkins botanical laboratory, for valuable suggestions in the conduct of the investigation, and for help in correcting the manuscript.

<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 482.

sectioning on the freezing microtome, collected from time to time at the same station.

A number of stains were tried and Ehrlich's haematoxylin was finally chosen. In addition, some root sections were stained in Haidenhain's and Delafield's haematoxylin, methylene blue, and Flemming's triple stains, and some small tissue fragments in safranin-malachite green. Ehrlich's haematoxylin did not give a very satisfactory stain, apparently because of the large amounts of tannin and free acids present in the material, but it gave better results than any other stain tried. At first it was thought that the difficulty was due to the fact that the chromic acid of the fixative was insufficiently washed out, although material was washed for 48 hours in running water. It was found, however, that sections cut from living material on the freezing microtome gave similar results. Hence, it was concluded that the trouble lay in the material itself and not in the treatment. Great difficulty was encountered in cutting embedded material owing to the presence of large numbers of crystals, presumably of calcium oxalate, in the parenchyma of both the crown and the veins of the leaf; to the great lignification of the vascular elements, particularly in the fibrous roots; to the abundance on the above-ground portions of greatly thickened, probably silicified hairs (pl. 1, C and D, and pl. 2, D); and to the difficulty in removing all sand lodged between the closely packed adventive roots. The series from which the leaf traces and much of the crown anatomy were studied were therefore treated for 48 hours in 52 per cent hydrofluoric acid and infiltrated by very gradual steps either in celloidin or paraffin. Paraffin sections of crown vascular elements were cut  $3\mu$  in thickness; roots, runners, leaves, etc.,  $7\mu$ ; sections in paraffin for the gross anatomy of the crown,  $15\mu$ ; and celloidin series for reconstruction,  $40\mu$ . Series of crowns more than 2 years old have not yet been obtained, this being left for future work. Satisfactory "dissections" of whole crowns have been obtained by boiling in caustic solution and later washing away the parenchyma with a jet of water.

### THE RUNNER

In commercial propagation the vast majority of strawberry plants come from runners and not from seed. The "runner" is a very much elongated diageotropic<sup>4</sup> stem and can easily be distinguished from the large adventive roots by its hollow cylinder of vascular tissue, which is covered by a thick layer of cortex and which surrounds a very large parenchymatous medulla. (Fig. 1.) The vascular cylinder has an extensive primary xylem composed of exceedingly large vessels arranged in distinct but closely approximated bundles. Each of these bundles is made up of wedges of primary xylem separated by quite irregular medullary rays of one to four cells in tangential thickness. (Pl. 3, E.) This primary xylem is the chief functional portion of the xylem throughout the comparatively short life of the runner. Outside this in old runners is found a considerable amount of secondary xylem made up of much smaller, more heavily lignified tracheids. The whole is admirably adapted

<sup>4</sup> The physiological reactions of these runners have not been studied as yet, as far as is known. It is therefore uncertain whether the horizontal position is merely due to the runner being too weak anatomically to support itself in an erect position or whether it is the result of an active tropism.

for carrying the large amounts of food and water required for the complete establishment of the daughter plant. The medulla is made up of large, loosely packed, thin-walled cells quite different from the storage pith of the crown. The phloem is well developed. (Pl. 3, E.) The outer portion of the pericycle of the runner (pl. 3, C)<sup>5</sup> shows a less extensive tangential multiplication than is characteristic of that of the adventive roots and of the crown proper.

The runner has greatly elongated internodes which are covered with short unicellular hairs interspersed with numerous multicellular glandular ones. The first node bears a solitary leaf which often remains rudimentary. Each such leaf has in its axil a bud potentially capable of development as a runner or crown. The arrangement of bundles is particularly clear at the nodes of a runner. The base of the leaf encircles the stem in an oblique spiral, one stipule being much shorter than the other. The three bundle traces (fig. 1) are located at about equal distances from each other around the stem. The middle one rises highest; the right-hand one is usually somewhat lower down, and the left-hand one is lowest of all. This order is characteristic of the leaf traces throughout the entire plant. Where the nodes are close together the first indication of separation of the traces for the next higher leaf can be observed, one close to each of the traces here mentioned (fig. 1), but the cylinder is broken only for a short distance, the leaf-bundle gap formed being quite short. The two lateral leaf traces swing toward the central one in the petiole sheath, and from them arise the several stipular bundles.

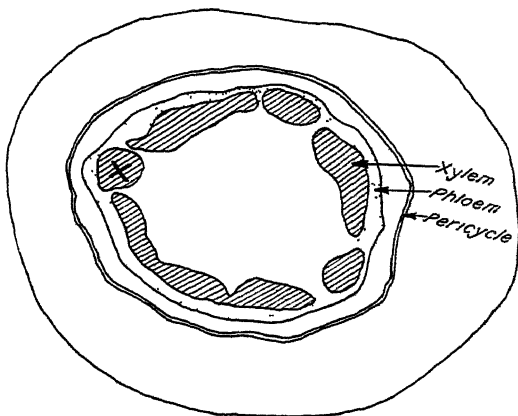


FIG. 1.—Transverse section of a runner near the node. The bundle at X is the median leaf trace of the leaf next above the position of this section. The xylem is well developed.  $\times 50$

After the runner has grown diageotropically for several centimeters its tip becomes negatively geotropic, turning up just beyond the second node and undergoing great thickening. It also puts forth adventive roots, and thus soon becomes established as a daughter crown.

The leaf which subtends each such crown remains attached often until its base is completely buried in the cortical parenchyma of crown and root. Its axillary bud sometimes remains dormant and is likewise buried with it in the parenchyma of the daughter crown. More often, however, this axillary bud continues to develop as a new runner. Since the growing tip of the primary runner, now that of the crown, has turned upward (pl. 1, B), the tip of this secondary run-

<sup>5</sup> Van Tieghem and Douliot (8) state that in the strawberry the pericycle of the roots may be of two or three layers, but that the innermost layers of the cortex undergo proliferation to form a periderm. They figure the endodermis as lying between two series of rectangular cells. What is termed "pericycle" in the present paper has not been tested microchemically for cutin, but there is no appearance of a distinct layer where, according to these writers, the endodermis should lie.

ner has all the appearance of being a continuation of the parent one. This appearance is enhanced by an anatomical peculiarity. The central trace of the leaf does not separate from the vascular cylinder of the runner below the origin of the bud, but remains as part of the



FIG. 2.—Longitudinal section of the vascular system of the tip of a runner, showing relation of crown, subtending bud, and leaf. The median trace of the leaf arises from the bud as if from a primary axis. The position of the lateral traces (figs. 3 and 4) shows the crown to be the true primary axis, the bud being axillary.

continuous cylinder well up on the outer side of the axillary bud, above its insertion into the base of the crown. A longitudinal section of the runner at this point through the median leaf trace would therefore show the leaf apparently arising from this bud as from a main axis. (Fig. 2.) It is only

the insertion of the other two traces into the daughter crown that proves this bud to be really an axillary and not a terminal one. (Figs. 3 and 4.)

### THE ROOTS

The primary runner does not give off roots below the leaf subtending the secondary runner. These arise solely from the crown proper.

The vascular system of the very base of the crown is a continuous inverted cone broken only by the two lateral leaf traces and, in those cases where a secondary runner develops, by the branch trace leading to it. (Pl. 3, D.)

If the axillary bud remains dormant, no branch trace is formed above it in the maturing vascular cone. If, however, this bud continues growth, as often happens, a decided gap is formed in the vascular cone of the crown. This gap does not, however, extend far up the crown. The



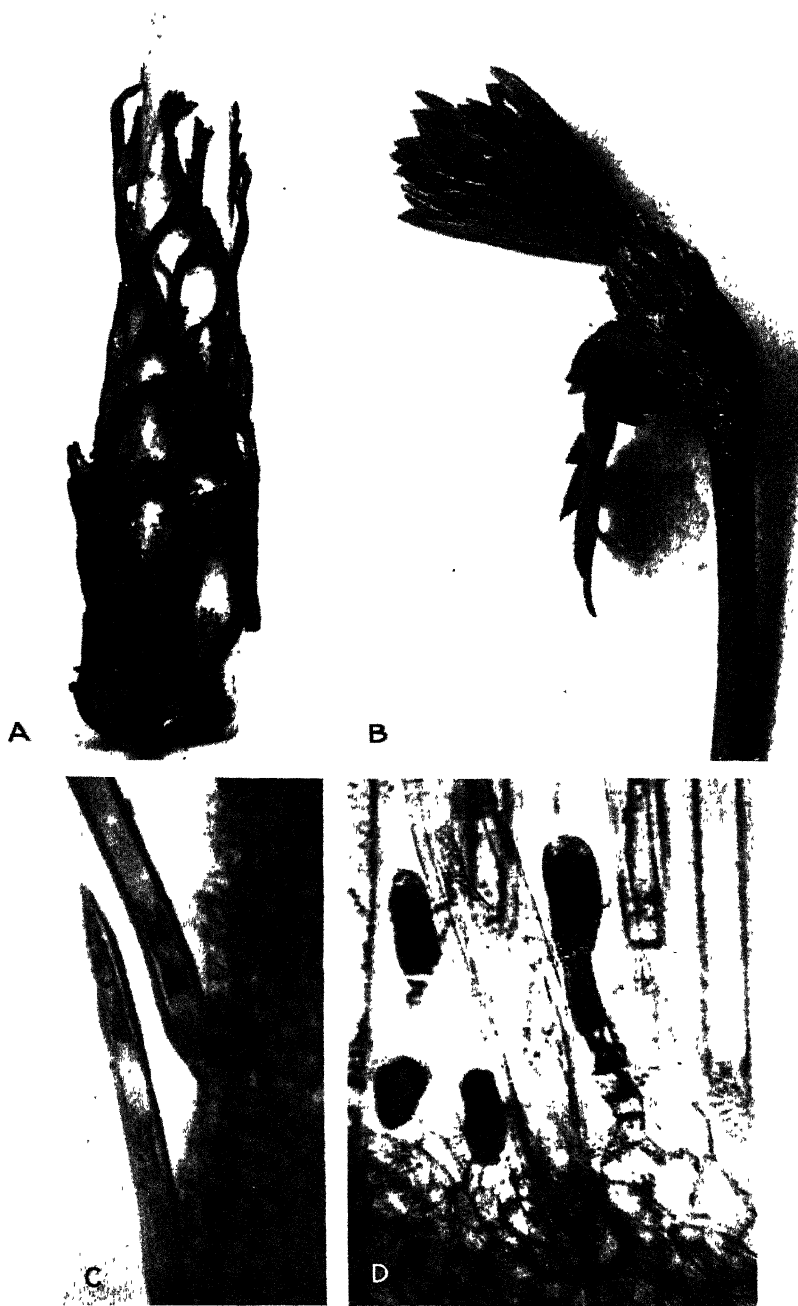
FIG. 3.—Longitudinal section of the vascular system of the tip of a runner, showing a lateral trace arising from the crown.



FIG. 4.—Diagrammatic representation of the arrangement of the three traces of the leaf which subtends a runner crown.

adventive roots arise directly from the pericycle just outside the surface of this vascular cone, or in older crowns from just outside its ramified vascular strands. They arise by tangential division of the cells of the multiple pericycle, accompanied by a differentiation of the tissues intervening between this and the vascular elements of the crown, into xylem and phloem elements. The root trace leaves no opening in the parent cone. The contact is made by individual groups of xylem cells of the crown. These roots are established on the younger portion of the crown, which, while its vascular system has a certain amount of mature xylem, is by no means completely formed. In most cases these roots do not undergo secondary thickening, and hence their xylem elements do not connect

with the later secondary xylem developed by the crown. Sometimes, however, the roots do become secondarily thickened. In such cases, as new xylem is laid down by the parent cylinder of the crown it is formed



A.—Dissection of old crown to show the vascular skeleton. The basal cone has been cut away and the youngest portions dissolved, leaving only the mature secondary xylem. Note spiral arrangement of the three main strands which are tied across by numerous narrower bands. The latter are also spirally placed but in the opposite turn.  $\times 3.75$

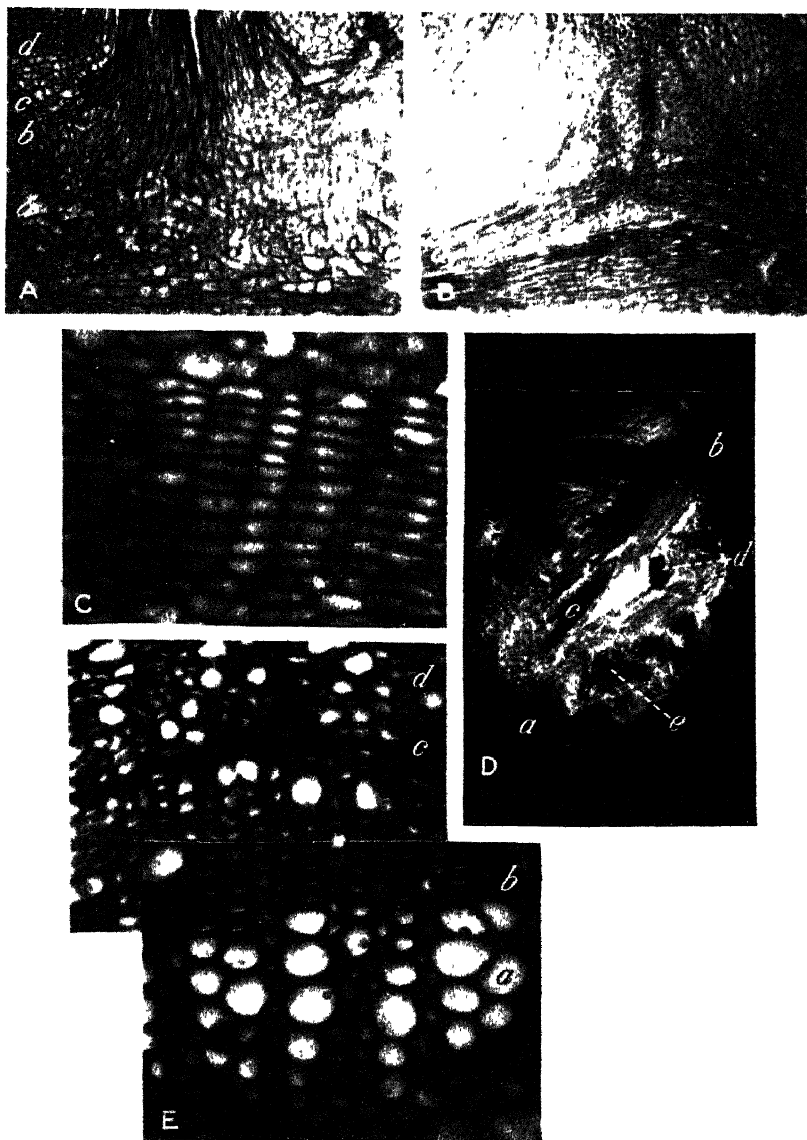
B.—Runner tip with the beginning of a new crown, before the formation of roots. The terminal bud has turned upward. Note the reflexed nodal leaf from the axil of which the runner will be continued.  $\times 2\frac{1}{4}$

C.—Longitudinal section of hairs from the upper part of the crown. The bases of the hairs are deeply embedded in the outer layer of cortical parenchyma.  $\times 375$

D.—The same with accompanying multicellular glandular hairs.  $\times 750$



- A.—Radial longitudinal section of a young xylem strand, showing the annular (a) and spiral (b) vessels of the primary xylem.  $\times 350$
- B.—Radial longitudinal section of a crown, showing a leaf-trace gap. The vascular tissue of the crown is broken off squarely above the gap.  $\times 88$
- C.—Radial longitudinal section of a crown, showing a branch gap. The vascular cylinder of the crown is broken only at the center of the daughter cylinder, no external gap being formed.  $\times 88$
- D.—Longitudinal section of the apex of a mature crown, showing the vascularization of young inflorescence. Note the protective hairs.  $\times 21$



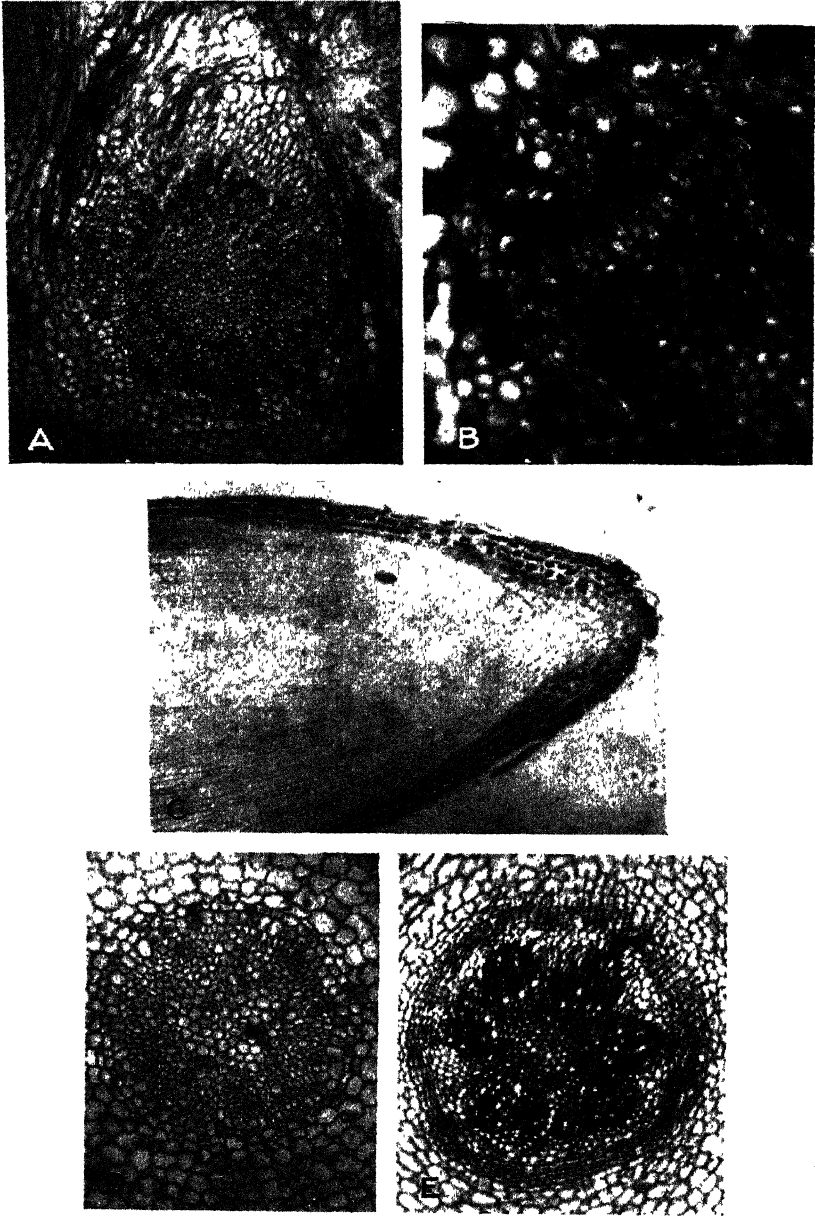
A.—Longitudinal section of a crown, showing connection of the various tissues of a young adventive root with those of the crown. *a*, Xylem; *b*, cambium; *c*, phloem; *d*, pericycle. Note particularly the continuation of the many-layered pericycle of the crown into the root.  $\times 125$

B.—Section as above but showing particularly the phloem (*a*).  $\times 125$

C.—Transverse section of runner, showing the many-layered pericycle or periderm. Note the large cells of the extrapericyclic cortex above. The parenchyma between the pericycle and the phloem is made up of much smaller cells.  $\times 250$

D.—The vascular system of the base of a young crown prepared by maceration. The photograph is taken from below. The parent runner is at *a* and the secondary runner at *b*. Note the bundle gap (*c*) of the medium bundle of the leaf subtending the secondary runner. The runner or branch gap (*d*) above is noticeably small in spite of the considerable development of the runner. Adventitious roots are to be seen at the sides. The gap at *e* is probably that of one of the lateral bundles of the leaf.  $\times 7\frac{1}{2}$

E.—Transverse section of the vascular tissue of a runner, showing primary (*a*) and secondary (*b*) xylem, cambium (*c*), and phloem (*d*). Note the size of the conducting vessels in the primary xylem.  $\times 250$



A.—Transverse section of adventitious root having an apparently polyarch stele and large medulla. The section is taken just at the level of the phloem in the crown so that the pericycle is not visible.  $\times 175$

B.—Transverse section of an irregularly pentarch root, showing the formation of an inter-fascicular cambium initiating secondary thickening. Note that here the cells of the medulla are also thickened to form a pseudovascular stele. These cells are, however, not greatly elongated as are the cells at the center of the steles of the fibrous lateral roots. Such cases are rare.  $\times 175$

C.—Longitudinal section of the growing point of an adventitious root. The various elements do not begin to differentiate until well back of the growing point. Compare with Plate 5, C.  $\times 70$

D.—Transverse section of an adventitious root taken just back of the growing point. The stele is here pentarch from the beginning.  $\times 350$

E.—Transverse section from an older similar root taken near its insertion into the crown. The stele is hexarch. Note the multiple pericycle and the relatively distant phloem strands.  $\times 175$

chiefly above the root and is connected to its secondary xylem. A thin area is thus left in the vascular cylinder of the crown just below the point of origin of the root. These areas are never large and appear to occur only below secondarily thickened roots.

These adventive roots, which give rise to the entire root system of all except seedling plants, arise apparently from any part of the central cylinder of the crown, and are very often found actually pushing through the bases of still functioning leaves. Van Tieghem and Douliot (8) state that these roots can arise only at the two sides of a median leaf trace. The xylem strand is already differentiated before the root has pushed out through the cortex of the crown. (Pl. 3, A and B.) Once established, however, it pushes out very rapidly, its tissues maturing slowly so that the vascular elements beyond the surface of the parent crown are not differentiated until the root has attained almost its full diameter. (Pl. 4, C.) The vascular strands are therefore widely separated. The stelar plan of these roots appears to be exceedingly varied. It is either tetrarch, pentarch (pl. 4, D), hexarch (pl. 4, E), or in a few cases, apparently polyarch (pl. 4, A). No actual transition between these types has been observed, but from the differences noted in different sections of a single root it seems possible that they may sometimes change from one to another in the same root. It is, however, perhaps significant that both tetrarch and pentarch steles have been found at the first visible differentiation behind the growing point. Hence, steles of more than four strands do not necessarily arise by transition from this type, but apparently are formed as such from the beginning. The steles of these roots (at least in the material at hand) are never heavily lignified.<sup>6</sup> The phloem is well developed (pl. 4, E) and the pericycle usually many layered (pl. 4, E). The endodermis is distinguishable only in the very early stages. (See footnote 5.) Transition to a cylindrical stele by the formation of an interfascicular cambium does occur, but it is not the rule. (Pl. 4, B.) The cortical and medullary parenchyma are extensive but thin walled, and are usually almost devoid of stored starch or other observable materials. There are, in the older portions, large numbers of cells filled with what appears to be tannin. (Pl. 4, B.) The contents of these have not yet been tested microchemically, however, so that their exact nature is uncertain.

These adventive roots push out very rapidly and, as is shown by examination of the root systems in older plants, may reach a length of 4 or 5 inches. (Pl. 6.) Darrow has found instances in which these roots developed to a length of 10 inches without branch or taper. He also reports cases in which such roots bore root hairs throughout their entire length. Such cases have, however, not occurred in the material examined in this study. In the writer's material they do not appear to form root hairs themselves but give rise to lateral branch roots which are the true absorptive organs.

These secondary roots differ markedly from the parent roots. Their mode of origin is essentially that described by Van Tieghem and Douliot (8) for rootlets of *Fragaria*. They have, however, a decidedly different stelar plan from the parent type. The primary

<sup>6</sup> A few cases of development of interfascicular cambium have been observed in which secondary thickening took place. Such secondary thickening was accompanied by a lignification of the medullary parenchyma cells to form a pseudovascular stele. These cells, however, were not elongated and were unmistakably different from the central vascular strands of the fibrous roots. (See pl. 4, B.)

vascular structure is already differentiated at a point close behind the root apex. The marked thickening ultimately shown by these roots is therefore chiefly secondary. It is often possible to distinguish the first spirals of vessels in the root at a point less than its own diameter from the apical meristem, and indeed at a point which is still covered by the short rootcap. (Pl. 5, C.) The vascular bundle in these roots varies from a minute strand having a single large central vessel (fig. 5) which, but for the two lateral rays of small vessels, one on either side, might persuade one that he was dealing with a monarch stele, through diarch (pl. 5, D) and triarch (pl. 5, E) to the tetrarch type (pl. 5, A). No examples of more than four xylem rays were found in these branch roots. The diarch and tetrarch are by far the most common. Roots have been examined for this feature in four species and five varieties of *Fragaria* with like results. The evascular strands are heavily lignified and completely fill the center of the root. (Pl. 5, A.) There is no medulla whatever here, such as is present in the adventive roots, and the lignification progresses outward until the xylem portion of the stele consists of a solid woody strand. The phloem is well developed. (Pl. 5, A.) The endodermis, unlike that of the parent root, has greatly thickened walls (pl. 5, A and D), which perhaps serve as a protection against the fungi that are usually present in the cortex. The pericycle shows no tangential multiplication whatever such as is found in the parent type, remaining as a single layer of cells. Outside of the endodermis the cells of the cortex are filled with starch and certain of them harbor a fungus, which may be of a mycorrhizal character.<sup>7</sup> These roots bear numerous root hairs which are in no way peculiar.

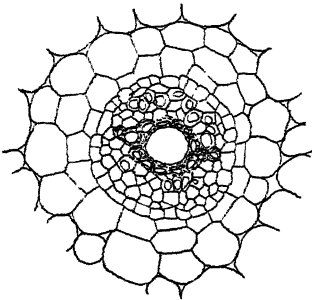


FIG. 5.—Transverse section of the stele of a fibrous root of *Fragaria elatior* which is diarch but has a single large central vessel.  $\times 250$

These two sets of roots make up the very extensive and efficient absorbing system of the strawberry crown.

### THE CROWN

The crown is at first a short fusiform body. It may later, by apical growth, become cylindrical and eventually fork into two or more divisions. The subterranean portion is covered with adventive roots. Aboveground it is clothed with leaves, the base of each of which closely encircles it for some three-fifths of its circumference. These leaf bases are closely imbricated and are provided with three distinct vascular strands each. The stipules are well developed and foliaceous. The petiole at its base is semicircular in cross section. The phyllotaxy is of the  $2/5$  type with a very much shortened spiral. Nowhere except in the runner does this spiral become much elongated.

As the leaves die their bases remain as dry, blackened scales. The three vascular strands persist long after the softer tissues have decayed, so that it proved quite easy to work out the overlapping of

<sup>7</sup> WHITE, P. R. MYCORRHIZA AS A POSSIBLE DETERMINING FACTOR IN THE DISTRIBUTION OF THE STRAWBERRY. [Unpublished manuscript.]

these bundles from such plants—much easier, in fact, than from serial sections. Working from below upward (figs. 6 and 7), the left-hand bundle of leaf No. 4 is just above the median bundle of leaf No. 1, while the right-hand bundle of leaf No. 5 lies above and on the same orthostichy as these two. The median bundle of leaf No. 6 lies directly above but a considerable distance from the median bundle of No. 1, so that two consecutive median bundles occupy positions in the order of imbrication,  $2/5$  of the stem circumference apart. In this way, two turns around the stem and five leaves are required between two leaves on the same orthostichy. The leaf traces themselves do not leave wide gaps (pl. 2, B), but above the branch trace of the axillary bud which is present over each median leaf trace there may be left a considerable gap (pl. 2, C), particularly when the bud develops to an inflorescence or a runner. The branch gap often becomes completely confluent with that of its subtending median leaf trace. It is, however, of later origin, the leaf trace being completely distinct before any indication of a branch stele becomes evident. (Fig. 8.)

These leaf bases, and more particularly the axillary buds and the structures subsequently developed from these, are densely covered with long, very much thickened, protective hairs. These hairs are unicellular, have a smooth outer surface and, except for the articulation with the stem or leaf surface, have a very small lumen as compared with their diameter. They are, however, quite thin walled at their bases. They originate as outgrowths of the cells of the epidermis, but their bases push inward so as to extend a short distance

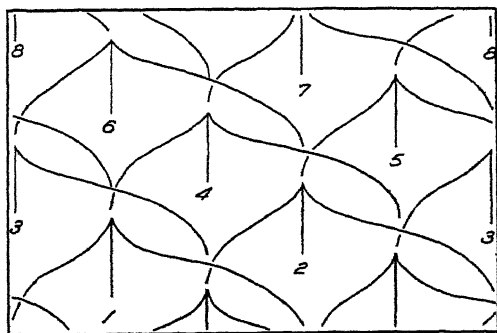


FIG. 6.—Diagrammatic representation of the arrangement of leaf traces. Since the bundles of one leaf do not connect directly into those of another, no representation has been made of the main vascular cylinder of the crown, but only of the bundles back to their connection into this cylinder

beyond the general inner limit of this tissue. (Pl. 1, C and D.) Such hairs are particularly numerous in the immediate angle between the leaf base and the stem, forming a fringe of longer hairs on the stem at the line of juncture. They are accompanied by lesser numbers of short, apparently glandular hairs, each of which is made up of several short cylindrical cells and an enlarged terminal one. (Pl. 1, D.) These glandular hairs may perhaps secrete a volatile oil, though the nature of their secretion has not actually been determined.

Aside from these emergences, the epidermis of the crown is made up of cells of four-angled or polygonal outline which show no apparent protective modifications. The function of protection is apparently entirely relegated to the leaf bases. No indication of suberization or cutinization was observed. Van Tieghem and Douliot (8) mention "liège" in speaking of the periderm, but it is uncertain as to just what they refer.

Below the epidermis in all regions of crown and roots there is a considerable primary cortex of large, quite closely packed, parenchyma-

tous cells, which in many regions, particularly the older parts of root or crown, show reticulate thickenings on the walls similar to those of the medulla and of the epidermis of the leaf. (Pl. 7, A.) No cork cambium was seen at any point, though Van Tieghem and Douliot (8) mention the occurrence of periderm here; and no bast fibers or other supporting tissues are present. In the entire plant, even to the leaves, the function of support seems to be served by the tracheids alone. Within the cortex, in the stele itself, the pericycle undergoes division, forming quite a large ring of thin-walled cells closely resembling those of a cambium. (Pl. 7, C.)

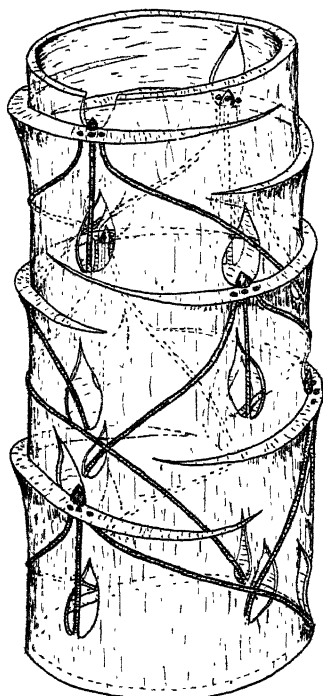
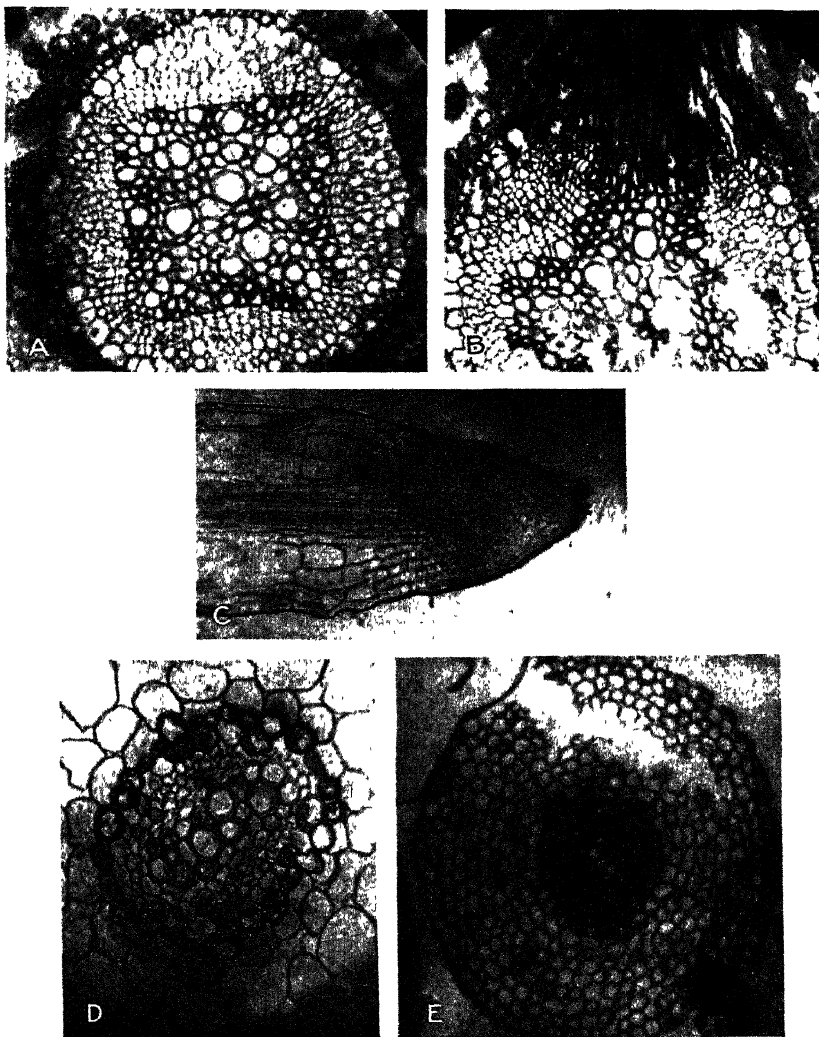


FIG. 7.—Leaf-trace arrangement, semi-diagrammatic. The bundle gaps are actually much larger than suggested by this figure. (See pl. 1, A.) Each leaf draws its water from three quite distant portions of the crown circumference

for the study of the vascular system the outer tissues peeled off in two layers, the first from epidermis to pericycle, the second from pericycle to cambium. The endodermis is quite indistinguishable except in the very early stages before the commencement of this division in the pericycle. Van Tieghem and Douliot (8) figure the endodermis as in the middle of the pericycle-periderm complex, but the writer has not been able to distinguish it in his material. Within the meristematic layers of the pericycle is a narrow region of parenchyma of somewhat smaller cells than that without. These are in part cells of inner layers of the pericycle and in part cells of the outer edges of medullary rays. The primary phloem strands are widely separated. The secondary phloem itself never becomes even a comparatively continuous cylinder except for a few layers which are broken into wedges separated by wide medullary rays. It appears to be made up of simple, thin-walled cells which are somewhat elongated and interspersed with very sparse companion cells. No sieve plates, either terminal or lateral, have been observed in any preparations, the phloem being distinguishable in longitudinal section from the parenchyma chiefly by its elongated, oblique-ended cells (pl. 7, D) and by its darker staining reaction. In a tangential section

the medullary rays which pierce it are made up of large, thin-walled rounded cells accompanied by somewhat smaller cells having dense protoplasts and large nuclei like those of the xylem ray shown in Plate 7, E. There is no indication of thickening or pitting in ray cells. The cambium is of few cells in thickness and of hardly sufficient regularity to constitute a definite ring or cylinder.

The leaf traces, as has been said, overlap in a perfectly regular manner. They do not, however, connect definitely with other traces, as is true in many herbaceous plants as well as in certain woody ones (3), but are fused into what, from its basal condition, we may interpret as a continuous woody cylinder broken only by the leaf and



A.—Transverse section of a fibrous root having a tetrarch stele. The primary and secondary xylem are clearly distinguishable by their staining reaction. Both fascicular and interfascicular cambiums are active and the phloem is well developed. The pericycle is unmodified, whereas the endodermis is greatly thickened. The innermost cortical layers are filled with tannin. Compare with Plate 4, A, D, and E.  $\times 160$

B.—Origin of a branch root from a fibrous root such as that shown in A.  $\times 160$

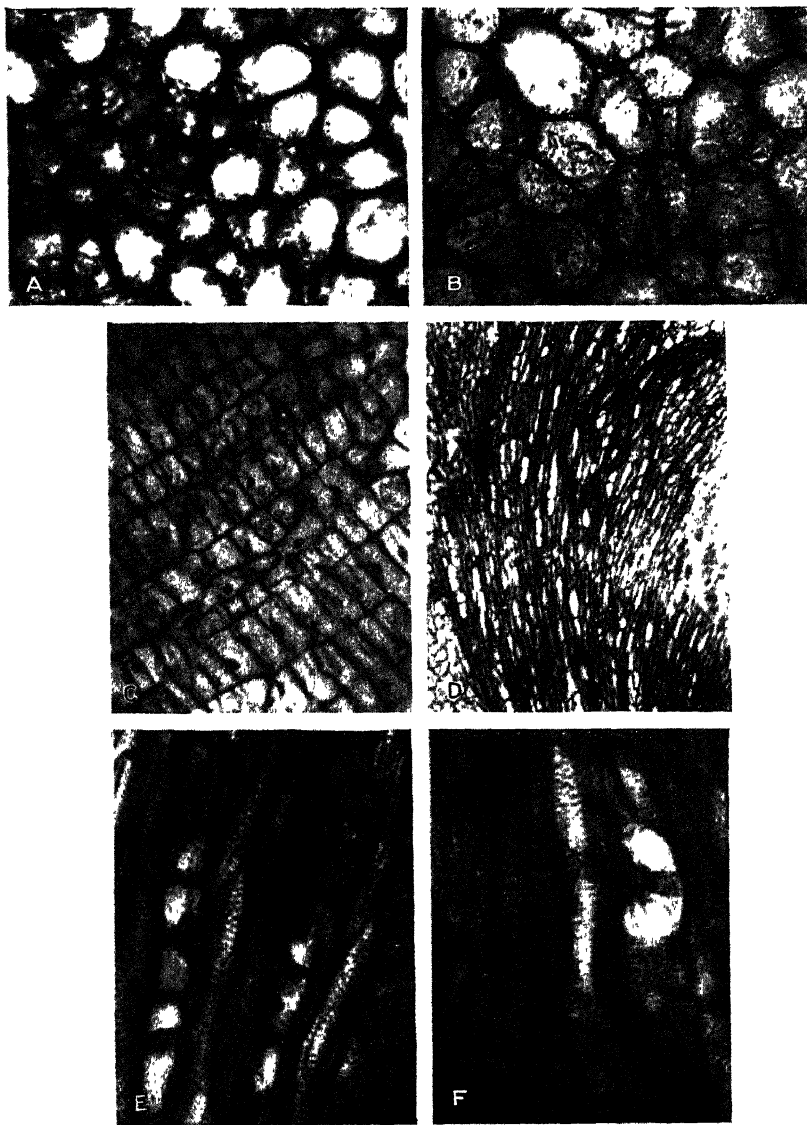
C.—Longitudinal section of the growing point of a fibrous root. The differentiation of vascular elements takes place very close to the apex. Compare with Plate 4, C.  $\times 160$

D.—Transverse section of a small fibrous root, showing the diarch stele. The endodermis and pericycle contain some tannin.  $\times 320$

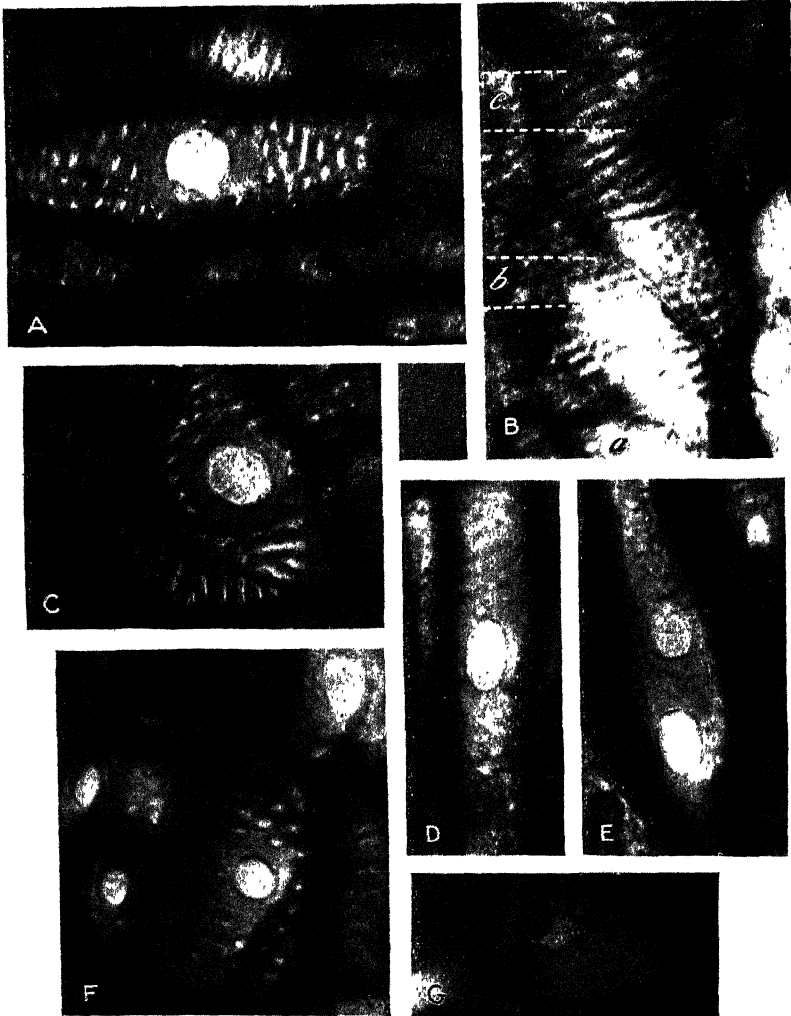
E.—Transverse section of a fibrous root having a triarch stele.  $\times 64$



entire strawberry plant. Note the stocky unbranched adventive roots (a) which give rise to slender fibrous ones.  $\times \frac{2}{3}$



- A.—Section of medullary parenchyma showing reticulate thickenings in the walls. Section taken from near the vascular cylinder.  $\times 250$
- B.—The same tissue nearer the center of the crown, where such reticulations are lacking.  $\times 250$
- C.—Transverse section of the multiple pericycle of the crown.  $\times 250$
- D.—Tangential section of the crown in the region of the phloem, showing the oblique-ended conductive cells and the narrow, few-celled medullary rays.  $\times 63$
- E.—Tangential section of crown xylem, showing detail of the medullary rays. The rays piercing the phloem are similar in structure.  $\times 500$
- F.—Radial section of secondary xylem. The pores in the tracheids are clearly in the lateral walls.  $\times 500$



A.—Radial section of xylem of the crown, showing a circular pore in the lateral wall of a short tracheid.  $\times 600$

B.—Tangential section of xylem, showing pores. The three pores at a, b, and c connect the short tracheids, forming an oblique conducting element.  $\times 600$

C.—Note the area, immediately around the large pore, which is free from pits. Note also the scalariform thickenings.  $\times 600$

D.—An elongated pore in the center of the lateral wall of an unusually long tracheid.  $\times 600$

E.—Section showing two pores in the same lateral wall of a tracheid.  $\times 600$

F.—Section showing the relative abundance of these pores.  $\times 600$

G.—Rings of bordered pits encircling pores in this manner appear to be very rare.  $\times 600$

branch gaps. This cylinder is not made up of uniformly longitudinal tracheids or tracheae (see footnote 8, p. 490) as are those of most woody stems. The strands are not simply parted at the gaps and fused again above. Bands in which the tracheids lie, in a tangential plane, perpendicular or oblique to the long axis of the plant are the rule rather than the exception. (Pl. 1, A.) It is only rarely, even immediately below a leaf or stem gap, that one obtains true transverse sections of the elements. In many cases narrow bands of xylem traverse horizontally what would otherwise be large gaps. (Pl. 1, A.) A very complex anastomosis thus arises which provides very efficient channels for a rapid and uniform distribution of material from any point throughout the circumference of the plant. The efficiency of this system is still further increased by the presence of large numbers of pits or pores similar in form to those found in the end walls of tracheal elements of many dicotyledonous plants. These pores are, however, found not only in the end walls but also in great numbers in the radial lateral walls of the tracheids, together with bordered or simple pits of the ordinary type. (Pl. 7, F.) They have not been found in the tangential walls of these elements. These pores are usually of a diameter of about two-thirds that of the cell lumen and show no trace of a middle lamella, even in sections cut from living material. (Pl. 8, B.) The pores are in most cases quite circular, but the occasional irregularities of outline and the rare occurrence of crossbars suggest the possible origin of the large pores by the fusion of smaller ones. The rarity of such irregularities and the entire absence of definite intermediate stages even in the xylem of very young crowns make it seem much more probable that the pore is due to the absence from the first of any secondary deposition of material on this area of the cell membrane and a breaking down of the middle lamella itself. Each pore is usually found in the center of an area of cell wall free from pits (pl. 8, A, C, D, and F), but a few cases were observed in which a distinct ring of bordered pits was present around such a pore (pl. 8, G). Cells have been found with more than one such pore in the same side (pl. 8, E), and others with pores in the opposite walls. Long series of cells have also been observed, in tangential longitudinal section, where pores in the lateral walls connect the whole series into one oblique continuous passage. (Pl. 8, B.) In this way, tangential circulation in the crown is not only provided by the peculiar arrangement of the vascular strands themselves and by the interweaving of the leaf traces, but a

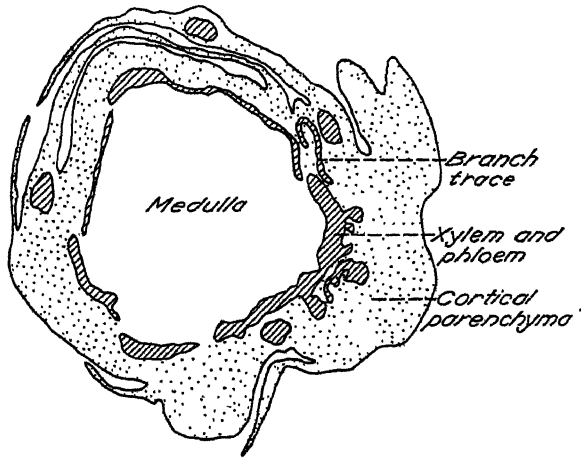


FIG. 8.—Transverse section of entire crown, showing relative proportions of the tissues. Compare with Figure 1 (runner).  $\times 10$

are in most cases quite circular, but the occasional irregularities of outline and the rare occurrence of crossbars suggest the possible origin of the large pores by the fusion of smaller ones. The rarity of such irregularities and the entire absence of definite intermediate stages even in the xylem of very young crowns make it seem much more probable that the pore is due to the absence from the first of any secondary deposition of material on this area of the cell membrane and a breaking down of the middle lamella itself. Each pore is usually found in the center of an area of cell wall free from pits (pl. 8, A, C, D, and F), but a few cases were observed in which a distinct ring of bordered pits was present around such a pore (pl. 8, G). Cells have been found with more than one such pore in the same side (pl. 8, E), and others with pores in the opposite walls. Long series of cells have also been observed, in tangential longitudinal section, where pores in the lateral walls connect the whole series into one oblique continuous passage. (Pl. 8, B.) In this way, tangential circulation in the crown is not only provided by the peculiar arrangement of the vascular strands themselves and by the interweaving of the leaf traces, but a

continuous circulatory system is provided, both longitudinally and tangentially within each individual xylem strand of the crown.

The vascular system of the strawberry plant (xylem at least) may be compared to a double-walled Sach's bell jar, in which universal diffusion of fluid materials in all directions is interrupted only by the leaf gaps and branch gaps. These pores are found in the xylem of all of the older portions of the crown, particularly in the regions of root origins. A cursory examination showed them to be present in the species *Fragaria virginiana*, *F. chiloensis*, *F. elatior*, *F. nilgerrensis*, and *F. vesca* horticultural var. *Monstrueux Caemeux*, and in the varieties known as Howard 17, Progressive, Dunlap, Klondike, and Rockhill. It is to be presumed, therefore, that this feature is of general occurrence throughout the genus. This condition seems clearly to explain the physiological phenomenon observed by growers that, unlike most plants, injury to or severing of the roots on one side of a crown has no more serious effect on that side than on other parts of the plant. It can be said without a possibility of doubt that no direct connection exists here between individual branch and root such as appears, from the work of Auchter (1), to occur in the apple. The strawberry plant has one continuous tangentially interlocking water-conducting system rather than a group of essentially distinct systems.

As is to be expected in a system with these peculiarities, true vessels<sup>8</sup> are not abundant. The tracheids of the xylem are exceedingly short and stout. The primary xylem, it is true, is made up of a few large annular vessels and some spiral ones (pl. 2, A) which are quite long. Such vessels, however, appear to be present only in the primary xylem. When secondary xylem is laid down, it is in the form of short tracheids only. No true vessels are formed. The pitting varies with the position of the elements from the simple scalariform reticulations of the earliest portions to the complex bordered pits with narrow, oblique slit openings of the mature type. All gradations exist between these two. The medullary rays, like those of the phloem, are but few cells thick in longitudinal and tangential dimensions, but the cells are quite large. They appear not to be provided with pits of any kind. (Pl. 7, E.)

Within the xylem the central medulla is made up of large cells. The walls of these show distinct reticulate thickenings leaving irregular pits between. (Pl. 7, A and B.) These pits become more prominent from the center of the medulla outward, suggesting that they may possibly serve to allow movement of stored food in the neighborhood of the xylem elements. Similar reticulations are also present in the cortical parenchyma of the crown and sometimes in the parenchyma of the adventive roots. Starch has not been observed in these cells and the nature of their contents has not yet been determined. It is perhaps noteworthy that the same type of reticulation is to be found in the lateral walls of the cells of the upper epidermis of the leaf. Among all these medullary cells are large numbers of crystals, probably of calcium oxalate. Such crystals are also found in the cortical parenchyma and in the tissues of the leaves adjacent to the bundles.

<sup>8</sup> Strasburger's (6) text defines tracheae as "wider or narrower tubes formed from a number of cells by the disappearance of their end walls," while tracheids are defined as "single cells with pointed ends, and are as a rule of narrow diameter. Their walls bear peculiar pits." According to these definitions no true vessels are formed in the secondary xylem of the strawberry. The structures herein described certainly represent a transition type between tracheids and tracheae, but they resemble the former much more closely than they do the latter.

## DISCUSSION

The studies reported here have shown certain structural characteristics of the strawberry which are fundamental to an understanding of the growth and development of this plant. The runners with their long internodes are true stems, although their vascular structure differs markedly from that of the short internoded crowns. These differences are closely correlated with the essential differences in function of the two types. Although the xylem is extensive in both types of stems, that of the runner is composed of large, open, true vessels arranged in parallel bundles which allow free longitudinal movement of the large quantities of water and nutrients necessary for the maintenance of the runner plant and its dependent plants. In the crown, on the other hand, the entire vascular cylinder is made up of a network of short, anastomosing bundles which provide an efficient means of rapid transfer of water and solutes across the stem. Moreover, these bundles themselves, instead of being made up of long vessels, are composed of short tracheids provided with numerous large lateral and terminal pores which further increase the efficiency of cross as well as longitudinal transfer of water. Such a crown structure makes it possible for plants which have had most of their roots cut by a hoe or insect, or destroyed by fungi, to supply all leaves, runners, and other parts uniformly with water and nutrients. Broadly speaking, moisture and nutrients supplied to one side of a row of plants can be assumed to supply both sides of the plant uniformly, in striking contrast to most woody plants. Reduction of any part of the root system affects the plant as a whole rather than one side alone.

The runner is commonly two nodes in length and is not continuous beyond its first daughter crown, the runner being continued by what is really the bud from the axil of its first leaf. The manner of insertion of the lateral bundle traces of this leaf establish this point. The bud in the axil of this first leaf, however, owing to the bending up of the tip of the runner, is placed so nearly in direct line with the vascular bundles of the parent runner that it is in an especially favored position as regards its supply of water and nutrients. Hence its development is commonly far more rapid than is the development of any of the buds in the axils of later leaves on the same plant.

Marked differences in the arrangement, development, and structure of the vascular tissues of the large adventive roots which arise from the crown, as compared with the small fibrous ones, together with the paucity of root hairs and freedom from the normal (?) fungus associations on them, account for their comparative inefficiency as absorbing organs. This apparently explains the difficulty in transplanting plants having many large roots but no fibrous ones as compared with those having at least a few small ones.

## SUMMARY

The most distinctive features observed in the strawberry (var. *Progressive*) are:

1. There are two types of roots; large adventive ones, and fibrous branch roots, which show the following perfectly distinct and characteristic morphological differences:

- a. The tissues of the adventive roots differentiate very tardily; those of the fibrous branch roots differentiate early.

b. The vascular bundles of the former are placed far apart; those of the latter are very closely approximated.

c. The former have a large central medulla; in the latter the central medulla is entirely wanting.

d. The former very rarely show secondary thickening; in the latter secondary thickening occurs abundantly.

e. The steles of the adventive roots are tetrarch, pentarch, hexarch, or polyarch; those of the branch roots are diarch, triarch, or tetrarch only.

f. In the former the pericycle is many layers in thickness; in the latter it is of a single layer.

g. In the former the endodermis is unmodified; in the latter the endodermis is greatly thickened.

h. The adventive roots are without definite fungus-bearing regions, while in the branch roots a mycorrhizal (?) fungus is commonly present in a definite region of the cortex.

2. There is a many-layered pericycle in the crown and in the adventive roots.

3. Numerous oblique and transverse conducting strands are present in the mature crown.

4. The tracheids are provided with highly peculiar pores in their lateral walls, which greatly increase the possibility of tangential movement of material within a single xylem strand.

The physiological importance of these features needs to be further examined experimentally, and the anatomical characters, particularly in regard to their occurrence in related genera, need further study.

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# COMPARATIVE STUDIES OF WINTER HARDINESS IN WHEAT<sup>1</sup>

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## THE WINTER-HARDINESS PROBLEM IN WHEAT

Winterkilling is practically the only factor which limits the growing of winter wheat in northern regions, especially in the north-central part of the United States. The losses due to winterkilling in the United States, the methods of reducing winter injury, and the hardiness of certain varieties have been shown previously (10, 11, 26, 28).<sup>3</sup>

Several difficulties are encountered in determining the hardiness of wheats by field methods. Winterkilling of wheat plants in the field is very irregular, and complete killing or complete survival of the plants frequently occurs. Repeated field tests are necessary to measure hardiness in varieties. Poorly adapted varieties often show a low winter survival even though they are known to be resistant to cold (14). Frequently it is not low temperatures, but drought, soil blowing, soil heaving, and smothering by ice which are responsible for the killing of winter-wheat plants. The low correlation between the survival of winter-wheat hybrid strains and that of selections from them was shown by Vassar.<sup>4</sup> In the writer's experiments no significant correlations were obtained between the survival of hybrid strains and selections from them. A laboratory method for measuring winter hardiness, which would be more accurate than field observation, therefore, is greatly needed.

The complexity of the problem and the differences between varieties have made positive conclusions regarding the nature of hardiness rather difficult. Many characters possessed by hardy wheat varieties also are found in nonhardy varieties or they do not occur in rye, which is harder than wheat. The hard red winter wheats are the hardiest group known. These wheats are characterized by narrow, dark-green, prostrate leaves in the seedling and rosette stages. The narrow leaves seem to be fairly well associated with hardiness in certain American winter wheats but not necessarily in all groups of wheat. The dark color and prostrate position of juvenile leaves also are not definitely related to hardiness. Any relationship between hardiness,

<sup>1</sup> Received for publication Dec. 2, 1926; issued November, 1927. A thesis submitted to the faculty of the Graduate School, University of Minnesota, in partial fulfillment of the requirements for the degree of doctor of philosophy, June, 1926. Most of the investigations reported herein were conducted at the Minnesota Agricultural Experiment Station, University Farm, St. Paul, Minn., during the years 1923-24 and 1925-26.

<sup>2</sup> The writer wishes to express his thanks to Dr. R. A. Gortner, professor of agricultural biochemistry at the University of Minnesota, for his help in outlining these experiments and in providing facilities for carrying them out; to Dr. H. K. Hayes, professor of plant breeding, and other members of the staff of the Minnesota Agricultural Experiment Station, for materials and equipment furnished and suggestions given during the course of these investigations; and to Dr. Robert Newton, professor of field husbandry at the University of Alberta, for supplying the writer with some of his experimental results on winter hardiness of wheat in advance of their publication by him.

<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 532.

<sup>4</sup> VASSAR, L. P. FIELD AND LABORATORY METHODS FOR A DETERMINATION OF WINTER HARDINESS IN WINTER-WHEAT HYBRIDS. Unpublished thesis, submitted to the Graduate School of the University of Minnesota in partial fulfillment for the degree of master of science, June, 1924.

all anatomical characters, and nearly all physiological characters probably could be disproved by the proper selection of a limited number of cereal varieties.

The recent work of Newton (34, 35, 36) shows, however, some important physiological characteristics of several of the hardy wheat varieties, which might be used in measuring the hardness of varieties and hybrids.

## REVIEW OF LITERATURE ON WINTER HARDINESS IN PLANTS

### CAUSES OF WINTERKILLING

In spite of abundant data to the contrary the belief of the old Greek philosophers and of Duhamel and Buffon as late as 1740 (13) that frost injury in plants is due to rupture caused by growing ice crystals within the cells has not yet been entirely dissipated. Göppert (15), Sachs (44), Müller-Thurgau (32, 33), Cavallero (reported by Abbe (1)), Molisch (30), and Wiegand (56) observed that ice formation takes place mostly in intercellular spaces and that the rupture of cells by freezing rarely occurs. Müller-Thurgau (32, 33) reported that ice formation takes place within the cell only on rapid cooling, and Cavallero (1) stated that, even though ice is formed within the cells, the protoplasm never freezes. Molisch (31), Müller-Thurgau (32, 33), and Wiegand (56) stated that frost injury was due to the withdrawal of water from the cells by freezing.

Other workers, including Cavallero (1) and Schindler (51), believed that frost resistance was due to chemical peculiarities of the cell protoplasm. Frost injury was believed by Gorke (16), Lidforss (24), Schaffnit (48), and Schander and Schaffnit (50) to be due to the precipitation of cell proteins, through salting out by the increased concentration of the cell sap after water had been withdrawn in freezing. Voigtländer (55) and Chandler (9) disagreed with this theory, the latter finding that plants grown with an abundance of nutrient salts are hardier and their protoplasm less easily precipitated than is the case with plants grown with a poorer supply of nutrients. The higher sap concentration of the better nourished plants, however, probably tends to increase imbibition; in which case Chandler's objection is not necessarily valid.

Mez (29) believed death to be due to cooling a plant below its specific minimum and held that the prevention of undercooling during freezing sometimes keeps the temperature of a plant above its specific minimum. Apelt (4) found that undercooling will not continue long, while Voigtländer (55) stated that undercooling rarely occurs in nature and is not related to the killing point or the death of the plant. Sachs (44) believed death from cold resulted from several factors, including shrinkage and disorganization of the cell following ice formation, ice expansion injury, and destruction by thawing after freezing. Detmer (12) stated that plant tissues were killed during freezing, because a change in the color of the chlorophyll could be observed.

The recent view appears to be that frost killing consists in the withdrawal of water from the cells to form ice in the intercellular spaces, followed by the precipitation of the protoplasm, due chiefly to the increased concentration of the cell sap.

## THE NATURE OF COLD RESISTANCE

The factors which frequently have been reported concerned with cold resistance include low moisture content, increased sugar content<sup>5</sup> (2, 3, 4, 18, 24, 36, 48), increased sap concentration, increased colloidal material, and a long vegetative period (8, 51), as well as certain morphological characters of wheat, such as narrow or prostrate leaves (8, 45) and large cells (5).

The relation of low moisture content to hardiness in wheat was shown by Sinz (53) and Newton (35). The same relationship was shown in apple twigs by Shutt (52) and by Beach and Allen (7), in peach twigs by Johnston (21), in plum twigs by Strausbaugh (54), and it has been indicated for vegetable plants by Rosa (43).

Although Maximow (27) and Chandler (9) found that increasing the sap density of plants increased the resistance to freezing more than could be accounted for by the increased osmotic pressure, Salmon and Fleming (46), Pantanelli (40), and Baroulina (5) found no definite relation between osmotic concentration of the sap and the cold resistance of cereals. The failure to find a relation probably was due to the selection of varieties, the attempt to compare different crops, or the time of collecting samples. The method of extraction influences the freezing point of the sap, and it may differ considerably from the freezing point of the tissues, as shown by Newton and others (38) and by Wright (58).

Imbibitional pressure due to hydrophilic colloids, as measured by the quantity of juice expressed from wheat leaves, was shown by Newton (34, 35) to be closely related to hardiness, the more hardy varieties yielding the lesser quantity of juice. The relation of total solids and bound water to hardiness was pointed out by Newton and Gortner (37).

Rosa (43) reported pentosan content to be related to hardiness in vegetable plants, but Newton<sup>6</sup> did not find this relationship in wheat. Recent experiments by McGinty (25) indicate that the methods used by Rosa would not actually measure pentosan content.

Zacharowa (59) reported the most cold-resistant tissues to be the most alkaline and to contain a lower salt concentration and a higher protein content. Kolkunov (23) reported that xerophytic wheat varieties resist injury from low temperatures.

Newton (35) suggested that, because of the better retention of sugars, hardy cereals may have a lower rate of respiration than non-hardy ones, which has been shown by Govorov (18) to be the case.

## LETHAL TEMPERATURES FOR CEREAL PLANTS

Schaffnit (49) found that wheat seedlings were injured very little at  $-5^{\circ}$  to  $-10^{\circ}$  C. but were considerably injured at  $-15^{\circ}$  to  $-20^{\circ}$  C. Both the degree and the duration of cold affect the extent of killing. Klages (22) exposed wheat plants to cold for short periods and obtained results similar to those of Schaffnit. Rye seedlings three or four days old were found by Zacharowa (59) to be killed at  $-7.8^{\circ}$  but not at  $-2.9^{\circ}$  C. At  $-5.75^{\circ}$  and  $-3.9^{\circ}$  C. the seedlings were killed except at the root tips. Buhler (8) froze the juices of spring

<sup>5</sup> NEWTON, R. THE NATURE OF WINTER HARDINESS IN CROP PLANTS. Progress Reports to the Honorary Advisory Council for Scientific and Industrial Research (Canada). (Typewritten reports, May, 1924, and May, 1925, furnished the writer by courtesy of Dr. Robert Newton.)

<sup>6</sup> NEWTON, R. Op. cit.

barley, winter barley, and winter rye and found at  $-7.0^{\circ}$ ,  $-12^{\circ}$ , and  $-15^{\circ}$  C., respectively, a precipitate consisting of protein with traces of lime and phosphoric acid.

### PROBLEMS INVESTIGATED

The chief object of the investigations undertaken by the writer was to determine the most accurate and feasible methods for measuring winter hardiness in wheat varieties and hybrids. Four general problems were studied: (1) Laboratory investigations of factors for hardiness; (2) determination of lethal temperatures for wheat; (3) hardiness of plant parts; and (4) hardiness in wheat hybrids. Other objects of the investigations were to determine further the processes of hardening, the causes of winterkilling, and the inheritance of winter hardiness in wheat plants.

### LABORATORY INVESTIGATIONS OF FACTORS FOR HARDINESS

The factors which were investigated in the laboratory studies included the moisture content and imbibitional pressure of the leaves, total solids, freezing-point depression, and bound water in the expressed juice or sap, the seasonal changes in these factors, and the rate of respiration of wheat varieties at low temperatures.

### MATERIALS AND METHODS USED

Four varieties of wheat—Minhardi, Kanred, White Winter, and Marquis—were used in these investigations. Minhardi has been shown (11) to be the hardest winter-wheat variety grown in the United States. Kanred, an important hard red winter variety, is less hardy than Minhardi. White Winter was selected because it appeared to be among the least hardy of the winter wheats. Marquis, a spring wheat, was used for comparison with the winter wheats because of its relative hardiness among the spring wheats. Swedish (Minnesota No. 2) rye was included in the experiments because it was known to be considerably more hardy than Minhardi wheat and to determine whether the same physiological factors for hardiness occur in wheat and rye.

The moisture content of the leaves was determined from samples of approximately 10 grams, weighed before and after drying 24 hours or more in an oven at  $100^{\circ}$  C. The imbibitional pressure of the leaves was determined by measuring the quantity of juice or sap per gram of green tissue expressed by a hydraulic press at pressures of 50, 150, 250, and 350 kgm. per square centimeter. Samples, usually consisting of 25 to 75 gm. of tissue, were wrapped in pieces of boiled unbleached muslin 5 inches square. They were then placed in a steel press bowl and the hydraulic pressure applied to a piston in the bowl.

The percentage of total solids in the sap was determined by means of the Abbe refractometer, the sugar scale being used as a basis for estimating solids, as suggested by Gortner and Hoffman (17). Freezing-point depressions were determined with a Beckmann apparatus, a salt-and-ice bath being used. The percentage of bound water in the sap was ascertained by the method of Newton and Gortner (37), by determining the freezing-point depression of the

sap before and after adding pure sucrose in a quantity equivalent to a molecular concentration of sucrose in the water of the sap. A quantity of sap containing 10 gm. of water, as determined by the percentage of total solids, was weighed out, 3.422 gm. of sucrose added, and the freezing-point depression then determined.

For example, a sample of sap showed a freezing-point depression of  $1.51^{\circ}$  C. With a quantity of added sucrose necessary to give a molar concentration, it had a freezing-point depression of  $3.86^{\circ}$  C., which, minus  $1.51^{\circ}$ , equals  $2.35^{\circ}$ , the excess freezing-point depression. Of this excess,  $2.085^{\circ}$  was due to the sucrose, which in solution forms sucrose hexahydrate. Then  $2.35^{\circ}$  minus  $2.085^{\circ}$  equals  $0.265^{\circ}$ , which is the excess freezing point due to water being bound by colloids in the sap so that it can not dissolve the sucrose. Dividing  $0.265$  by  $2.35$  and multiplying the result by  $89.2$  gives  $10.1$  per cent of bound water. The figure  $89.2$  shows the quantity of free water in  $100$  c. c. of a molar solution of sucrose in which  $6$  molecules or  $10.8$  per cent of the water is taken up to form sucrose hexahydrate.

The lethal temperatures for wheat plants were determined by subjecting the plants growing in pots to freezing, usually for  $24$  hours at known temperatures, and then returning the pots to the greenhouse and noting the percentage of plants which recovered. The rate of respiration was measured by drawing a stream of air free from carbon dioxide through a closed chamber covering the wheat leaves, which chamber was sealed off from the laboratory air and from the soil in which the pots were growing by a half-and-half mixture of vaseline and liquid paraffin. The air was caused to flow through the chamber by equal suction and pressure at the rate of  $18$  liters per hour. Five volumes of air passed through the chambers during each half hour of aspiration, as the capacity of the chambers was  $1.8$  liters. The chambers were aspirated for half-hour periods whenever a sufficient quantity of measurable carbon dioxide had accumulated. The carbon dioxide given off by the plants was collected in bead-absorption towers containing a standard solution of barium hydroxide, and the excess of alkali was titrated with  $\text{HCl}$ , using phenolphthalein as an indicator. The acid solution was of such strength,  $N\ 0.04545$ , that each cubic centimeter was equivalent to  $1$  mgm. of  $\text{CO}_2$ . The temperatures during respiration were kept constant to within about  $0.1^{\circ}$  C. by placing the potted plants in the chambers of an ice-cream cabinet which were electrically heated by means of lamps, to keep the temperatures in the chambers above that of the surrounding bath. The temperatures were controlled by thermoregulators. (Fig. 1.)

The wheat used in these investigations of the tissue-fluid properties was sown in  $8$ -foot rows on fall-plowed land. The sowing was done by hand, September  $11$  and  $12$ , in  $1923$ , at the rate of  $5$  gm. per row or  $1$  bushel per acre. In  $1925$  it was sown with a hand planter on August  $27$ , at the rate of  $5.5$  gm. per row. The rye was sown September  $21$  in  $1923$ , but in  $1925$  it was sown on the same date as the wheat. Duplicate plots were sown consisting of  $25$  rows of each variety in  $1923$  and  $50$  rows in  $1925$ . In  $1923$ , delayed rains caused a slow growth of wheat, and the plants had reached only the three-leaf stage when the first samples were gathered October  $19$ . Growth continued, however, until the latter part of November. In  $1925$ ,

much better early growth occurred, but there was not much increase in dry matter after October 15. Heavy wrapping paper was placed

each year over the wheat plants after they had stopped growing to keep the snow from them. This facilitated the gathering of samples when the ground was covered with snow.

Duplicate samples of each variety were collected from adjoining rows at intervals of three or four days during the autumn and at greater intervals after December 15. Nearly all of the samples were collected between 10 a. m. and noon. The plants were cut off at the surface of the soil with shears and placed in screw-top bottles. They were then taken to the laboratory, and about 10 gm. of tissue was removed from the top of each bottle for moisture determinations. The remainder of each sample was weighed and taken immediately to the "hardening" room, located in the dairy building, where it was maintained in a frozen condition. Samples which were frozen when collected were not permitted to thaw. The hardening room varied in temperature usually between  $-12^{\circ}$  C. ( $10^{\circ}$  F.) and  $-23^{\circ}$  C. ( $-9^{\circ}$  F.) but dropped to the latter figure nightly. After they had been left 24 hours or more in the hardening room, the samples were

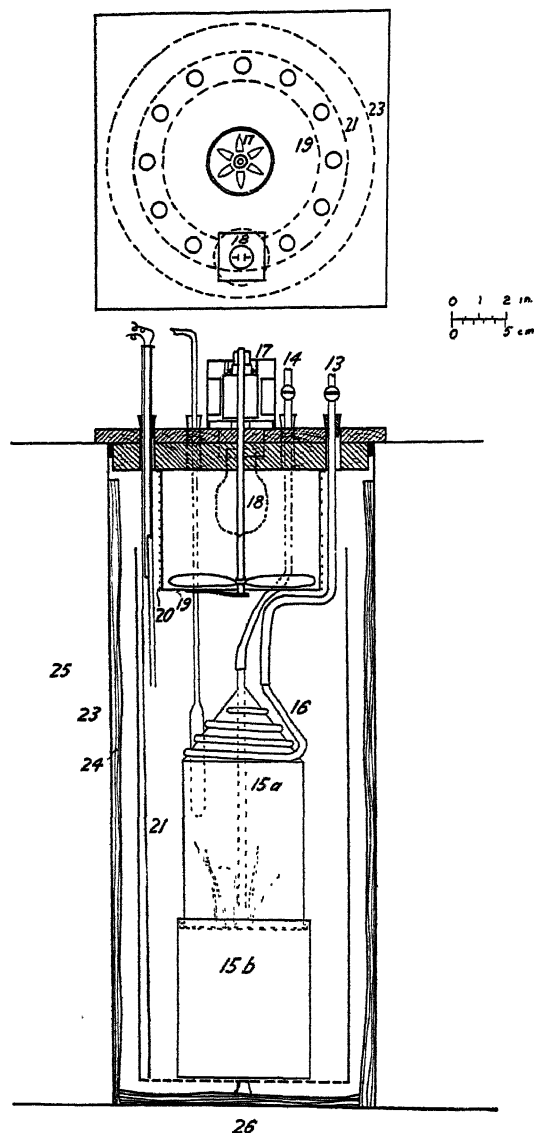


FIG. 1.—Diagram of the constant-temperature apparatus used for the respiration experiments: 13, Tube for ingoing air; 14, tube for outgoing air; 15a, sheet-metal respiration chamber; 15b, earthenware jar containing growing plants with the soil surface sealed off; 16, coil of copper tubing for cooling air before it enters respiration chamber; 17, fan motor; 18, socket and light for supplying heat; 19, support for fan shaft bearing and fan guard; 20, cylindrical wire screen surrounding the fan blade; 21, sheet-metal cylinder; 23, wall of cell of freezing cabinet; 24, asbestos lining for insulating the cell; 25, fluid of the freezing cabinet; 26, cork insulation of freezing cabinet

returned to the laboratory and pressed. In 1923, one of the duplicate samples of each variety was pressed soon after it was collected and

without preliminary freezing. In 1925, both samples of each variety were frozen.

The daily maximum and minimum temperatures recorded at St. Paul, Minn., during the fall and winter months from October to February, inclusive, in 1923-24 and 1925-26, are shown in Table 1. These data are given because of their relation to the hardening-off process in the wheat plants under field conditions, this relation being reflected in the data obtained on the different sampling dates as hereafter given. In 1923 the first freeze occurred on October 19, the date of the first collection of winter wheat from the field, but the wheat plants apparently had not been frozen. In 1925 the first collection was made on October 12, after light frosts had occurred, but while the plants were still in an actively growing condition.

TABLE 1.—*Daily maximum and minimum temperatures at University Farm, St. Paul, Minn., during stated fall and winter months of 1923-24 and 1925-26*

Day	Temperature (° F.)																	
	1923-24									1925-26								
	Oct.			Nov.			Dec.			Jan.			Feb.			Oct.		
	Maximum	Minimum		Maximum	Minimum		Maximum	Minimum		Maximum	Minimum		Maximum	Minimum		Maximum	Minimum	
1.....	66	47		52	23		43	19	-1	-19		32	28		57	43	50	27
2.....	72	42		45	40		35	11	10	-3		41	29		63	48	50	28
3.....	71	44		52	35		43	23	-2	-19		35	26		59	44	48	33
4.....	61	42		48	28		44	27	-9	-22		26	9		52	42	53	29
5.....	59	36		52	30		44	16	-6	-27		11	3		56	37	40	24
6.....	62	38		58	32		40	26	36	-6		12	3		51	31	31	19
7.....	62	44		43	26		45	26	31	16		20	3		41	38	25	12
8.....	59	41		41	22		45	29	32	22		26	1		42	37	29	8
9.....	75	48		58	31		33	21	30	20		28	18		39	30	49	23
10.....	77	52		59	37		35	20	23	15		37	12		45	26	52	30
11.....	71	54		60	31		48	21	15	6		33	24		59	41	56	33
12.....	58	43		49	41		42	17	6	-8		32	17		56	36	52	27
13.....	53	39		50	45		25	10	5	-11		39	27		58	43	47	30
14.....	52	34		48	43		41	14	13	-14		37	18		52	42	32	30
15.....	65	35		45	38		47	30	24	8		16	3		58	37	32	27
16.....	66	53		38	30		46	33	8	-16		26	12		44	32	30	22
17.....	69	53		55	28		52	32	6	-16		25	17		44	27	49	22
18.....	53	37		47	33		54	28	10	-1		22	14		33	29	50	34
19.....	44	30		50	28		49	34	2	-9		24	16		38	30	45	32
20.....	47	30		51	37		43	38	-6	-20		23	10		35	36	61	34
21.....	52	27		38	20		44	35	14	-21		16	2		47	26	45	26
22.....	55	31		37	16		36	28	34	14		21	-2		32	27	26	16
23.....	60	34		36	28		33	23	17	1		23	5		37	31	36	16
24.....	55	36		41	28		33	23	29	0		27	6		41	32	43	27
25.....	42	37		50	33		35	25	0	-15		36	11		42	30	37	23
26.....	44	39		33	26		42	26	0	-11		43	16		37	25	38	16
27.....	42	34		31	19		35	13	22	-8		44	19		26	26	18	5
28.....	43	32		41	19		26	9	36	17		39	26		24	17	21	5
29.....	34	22		46	27		20	8	40	24		33	28		23	13	23	19
30.....	32	19		46	23		8	0	37	23		27	8		31	16	1	14
31.....	44	24					11	0	35	28					42	24		32
Mean...	56.4	38.0		46.7	29.9		38.0	21.5	15.8	-1.7		28.5	13.8		43.9	31.5	40.0	23.2

The temperatures in 1923 continued rather mild, and the wheat continued to grow until well into November and resumed growth again during the few days following December 15. During the night of December 26 the temperature dropped abruptly and continued low until January 6, 1924. During this period the growth

of wheat stopped and the plants remained frozen and were covered with snow. The sudden change of temperature caught the plants in an unhardened condition, but in spite of this they survived the minimum temperature of  $-33^{\circ}\text{C}$ . ( $-27^{\circ}\text{F}$ .) of January 5 with little or no injury. This was due to the 3-inch covering of snow. Thermo-

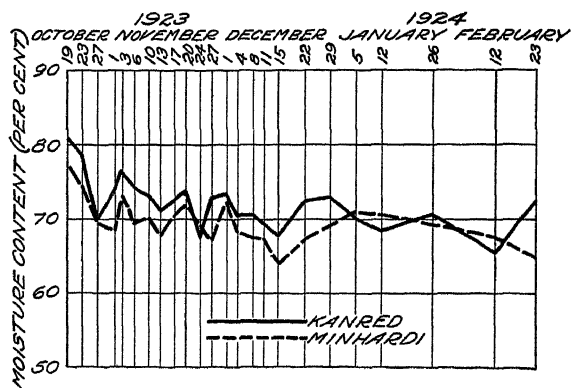


FIG. 2.—Moisture content of Kanred and Minhardi wheat leaves collected at intervals during the autumn and winter of 1923-24, University Farm, St. Paul, Minn.

graph records of the air temperature and the soil temperature just below the surface on a plot a few rods from the wheat plants were kept by A. C. Army, and some of the results were shown to the writer. During the first two weeks of January, when the air temperature dropped to a minimum of  $-33^{\circ}\text{C}$ . ( $-27^{\circ}\text{F}$ .) and rose to a maximum of about  $+2^{\circ}\text{C}$ . ( $+36^{\circ}\text{F}$ .), the soil temperature remained practically constant at  $-2^{\circ}\text{C}$ . ( $+28^{\circ}\text{F}$ .), which

is not fatal to any variety of winter wheat. After January 1 the wheat remained covered with some snow until spring.

In the autumn of 1925 the wheat apparently continued growth until the latter part of October and remained vegetatively active until the last week in November. The temperatures during December and January were relatively low, and there was some snow on the ground continuously. The wheat plants remained frozen nearly all of the time from the latter part of November until early in March, although the plants collected on January 16 had thawed somewhat.

The official temperatures recorded were taken with instruments placed in a shaded inclosure, while in the field on clear days the sunshine frequently caused the plants to thaw and take up water from the soil, even when the maximum temperature recorded in the instrument shelter was below freezing. The wheat plants thus were able to recover water to replace that lost by freezing and evaporation, apparently changing their imbibitional power during periods in which, on the basis of recorded temperatures, they would be expected to remain frozen and unchanged.

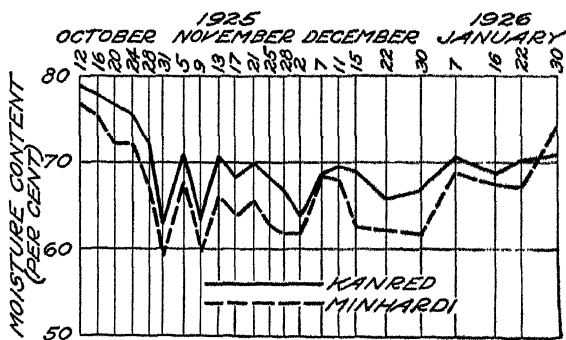


FIG. 3.—Moisture content of Kanred and Minhardi wheat leaves collected at intervals during the autumn and winter of 1925-26, University Farm, St. Paul, Minn.

The winter of 1923-24 might be considered an abnormal one, on account of the long-continued growing weather and the high temperatures in December, followed by very low temperatures in early January. The absence of low temperatures except when the ground was covered with snow permitted almost complete survival of winter wheat. The season of 1925-26, on the other hand, was more nearly normal, as temperatures tended to decrease gradually until the soil was frozen and covered with snow at the end of November. The conditions prevailing during that season and their effects on winter wheat were about such as might be expected in most seasons in sections having a climate similar to that of St. Paul, Minn. Rather heavy winterkilling occurred in the winter-wheat nurseries.

#### RESULTS OBTAINED

The data obtained from the laboratory investigations of factors for winter hardiness in wheat are shown in detail in the following pages. In the discussion following the presentation of these and other experimental results, the relations of the various factors concerned with hardiness and hardening are considered.

#### MOISTURE CONTENT

Data showing the average moisture content of duplicate samples of wheat leaves collected during the two seasons are given in Table 2 and are shown graphically in Figures 2 and 3. The moisture content of the leaves of the different varieties was about 80 per cent during early October, when the plants were actively growing. With the approach of cold weather the moisture content dropped to about 70 per cent. Most of this decrease resulted from the wilting of the tissues following freezing. After each freeze some of the lost moisture was recovered. This explains the wide fluctuation in moisture content shown in Table 2. Dead tissues were not entirely eliminated from the moisture samples. The separation of the dead tissue is very difficult when considerable quantities of partly wilted tissue, are present. It is not known just how little moisture the tissues could contain without being wilted in appearance, but, in general, all samples collected in late autumn or winter which showed less than 68 to 70 per cent moisture were at least partly wilted. The hardy varieties could contain less moisture than the less hardy varieties without being wilted.

From the data of Table 2 it is evident that the Kanred variety had a higher moisture content than the Minhardi on all but five dates. These exceptions followed severe freezes in which Kanred was more injured than Minhardi. The difference in moisture content may well be considered a measure of hardiness in which the harder variety has the lower moisture content. White Winter wheat, when not too badly wilted, showed a higher moisture content than either Kanred or Minhardi. Rye tends to have a high moisture content, even though it is extremely hardy. This, however, does not disprove the relationship in wheat, which belongs to another genus.

TABLE 2.—*Moisture content of the plants of four varieties of wheat and one variety of rye collected on stated days during the seasons of 1923-24 and 1925-26, University Farm, St. Paul, Minn.*

Date collected	Moisture content (per cent)				
	Minhardt	Kanred	White Winter	Marquis	Minnesota No. 2 rye
<i>Season of 1923-24</i>					
October:					
19.....	77.8	81.1			
23.....	74.7	78.9			
27.....	69.6	69.8			
November:					
1.....	68.6	73.7			
3.....	73.2	76.9	76.0		76.3
6.....	69.7	74.6			
10.....	70.2	73.4	72.6		75.2
13.....	67.8	71.3			
17.....	70.6	72.9	73.6		74.2
20.....	71.9	73.9			
24.....	69.1	67.8	73.4		69.6
27.....	66.9	72.8			
December:					
1.....	72.5	73.6	73.2		72.0
4.....	68.7	70.9			
8.....	67.5	70.9	69.1		
11.....	67.4	69.7			
15.....	64.2	67.9	57.3		
22.....	67.2	72.5	67.5		
29.....	69.2	73.1	72.7		
January:					
5.....	71.1	70.3	66.7		
12.....	70.7	68.5	68.6		
26.....	69.4	70.8	75.2		
February:					
12.....	67.7	65.7	69.2		
23.....	65.0	72.4	64.5		
March:					
8.....	56.7	63.7	64.3		
27.....	60.9	57.0			
May:					
17.....	75.1	78.7			
Average <sup>a</sup> .....	{ 68.677 ±0.512	{ 71.219 ±0.590	{ 69.593 ±0.817		
<i>Season of 1925-26</i>					
October:					
12.....	76.8	78.7	81.2	81.8	80.2
16.....	75.4	77.5	80.5	80.9	78.4
20.....	72.2	76.7	78.4	78.5	77.1
24.....	72.1	75.5	77.2	77.0	74.8
28.....	67.1	72.1	73.9	69.5	72.1
31.....	59.3	62.9	61.0	65.4	61.9
November:					
5.....	67.8	70.8	71.0	71.8	72.7
9.....	59.6	63.2	64.5	57.6	65.5
13.....	66.0	70.8	70.5	66.1	71.1
17.....	63.7	68.1	68.1	60.1	68.1
21.....	65.6	69.9	69.8	68.4	70.0
25.....	62.7	68.1	65.5	60.7	71.1
28.....	61.7	69.9	66.2	62.4	70.7
December:					
2.....	61.9	63.8	62.0	59.2	67.7
7.....	68.5	68.7	66.0	64.7	71.9
11.....	68.0	69.7	68.6	69.1	72.6
15.....	62.6	69.0	65.1	61.2	73.1
22.....	62.3	65.8	62.3	62.2	71.3
30.....	61.8	66.6	57.5	58.7	70.1
January:					
7.....	68.8	70.6			72.5
16.....	67.4	68.8	65.1	66.3	69.1
22.....	67.0	70.2			68.6
30.....	74.0	70.9	76.2	69.2	67.9
May:					
4.....	73.5	77.7			79.2
Average <sup>b</sup> .....	{ 66.635 ±0.682	{ 69.796 ±0.576	{ 69.171 ±0.933	{ 66.767 ±1.071	{ 71.409 ±0.518

<sup>a</sup> Not including samples collected Oct. 19.<sup>b</sup> Not including samples collected May 4.

The data on moisture content for 1925-26 are more complete than those for 1923-24. In 1925, on October 12, 16, 20, and 24, the moisture contents of Marquis and White Winter were distinctly higher than those of the hardier wheats, Minhardi and Kanred. Rye again showed a moisture content similar to that of the nonhardy varieties. During the autumn of 1925 the moisture content of all varieties decreased until about December 2, when a certain recovery occurred. The moisture contents of White Winter and Marquis leaves were relatively low after October 28. During this season the moisture contents again showed large fluctuations due to freezing and thawing, with consequent wilting and recovery. The higher moisture content of Kanred, as compared with Minhardi, was maintained throughout the season except on January 30. It is evident from this and from the behavior of White Winter and Marquis wheats that varieties somewhat similar in hardiness tend to fluctuate together, while widely different varieties are dissimilar because they are affected in widely different degrees by freezes or by their ability to recover. True comparisons in moisture content can be obtained only when the plants are not affected by freezing and wilting. On October 12, 16, and 20, the four wheat varieties had moisture contents in the inverse order of their hardiness. After that time this relationship did not exist. The lowest moisture contents of the nonhardy varieties were less than the lowest moisture contents determined for the hardy varieties, because the former contained more wilted and dried leaves.

Duplicate samples of 10 varieties of winter wheat and one variety of rye were collected for moisture determinations, from nursery rows, on May 31, 1924. The plants at that time had almost reached the jointing stage. The moisture-content data are given in Table 3 with the wheat varieties arranged in the order of their hardiness. Rye, although much hardier, had a moisture content higher than any of the wheats. The four hardiest wheat varieties—Minhardi, Buffum No. 17, Minturki, and Odessa—had moisture contents lower than the less hardy varieties, although the order of the moisture contents and the relative hardiness of these varieties among themselves was inverse to that of all varieties as a group. The remaining varieties with somewhat higher moisture contents also failed to show a definite relation between hardiness and moisture content. Differences in earliness and consequent stages of growth, however, may have affected the results.

On November 16, 1925, triplicate samples of five varieties of wheat were collected from the border rows of the varietal plots at University Farm. Collections were not made early in October, because sufficient growth had not been attained, and the samples collected November 9 were too badly wilted to show reliable results. The moisture contents of the five varieties, listed in their order of hardiness, are shown in Table 3. While again no definite relation between hardiness and moisture content were shown, Red Rock, the least hardy variety, contained decidedly more moisture than any other variety, and Kanred, the second least hardy variety, showed the second highest moisture content.

TABLE 3.—Average moisture content of samples of the plants of varieties of winter wheat and rye collected at University Farm, St. Paul, Minn., and listed in the order of average hardiness

Duplicate samples collected May 31, 1921				Triplicate samples collected Nov. 16, 1925	
Variety	Moisture content	Variety	Moisture content	Variety	Moisture content
	Per cent		Per cent		Per cent
Minhardi.....	74.9	Kanred.....	76.1	Minhardi.....	79.0
Buifum.....	73.4	Kharkof.....	76.7	Minturki.....	77.9
Minturki.....	73.3	Blackhull.....	76.4	Turkey.....	77.2
Odessa.....	72.7	Nebraska No. 28.....	75.5	Kanred.....	79.2
Padui.....	76.9	Minnesota No. 2 rye.....	81.2	Red Rock.....	82.1
Turkey.....	75.7				

An experiment to determine the rate at which wheat plants dry out at low temperatures was conducted by the use of artificial refrigeration. At the beginning of the experiment, samples taken from pots of Kanred and Minhardi wheat growing in the greenhouse contained

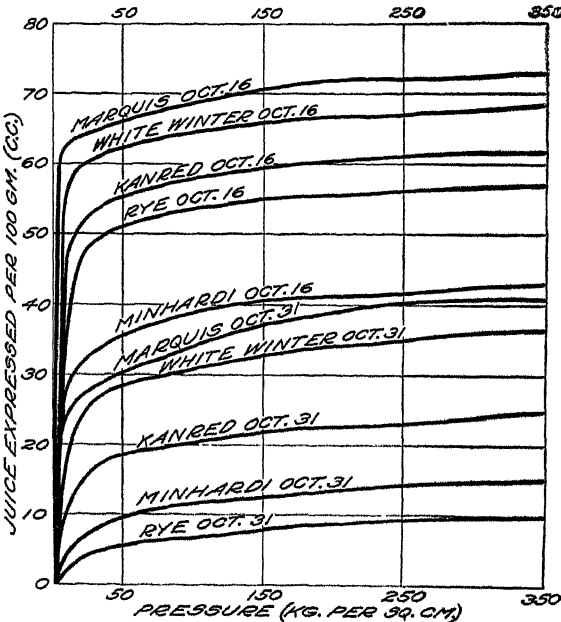


FIG. 1.—Quantities of juice expressed per 100 grams of tissue from frozen leaves of four varieties of wheat and one variety of rye at pressures of 50, 150, 250, and 350 kgm. per square centimeter, on October 16 and October 31, 1925, University Farm, St. Paul, Minn.

75.5 per cent and 73.4 per cent of moisture, respectively. Other pots containing plants of these varieties were then exposed successively to the following temperatures during the periods named: 1° C. for 24 hours, -8° C. for 36 hours, and -12° C. for 8 hours. At the end of this period Kanred contained 73.3 per cent and Minhardi 72.8 per cent of moisture. The remaining plants were then maintained at temperatures between -2° and -4° C. for one week. Under these conditions the plants were thawed, but the soil remained frozen. At the end

of the week the moisture content of the Kanred samples was reduced to 36.6 per cent and that of the Minhardi samples to 41.6 per cent. These latter samples of the two varieties consisted of the remaining plants from the same pots from which the previous samples had been taken. Duplicate pots of Kanred were returned to the greenhouse after the exposure at -12° C.

All of the Kanred plants and half of the Minhardi plants had been killed by this exposure. The subsequent rapid drying was largely

in dead tissue. Under usual freezing conditions most of the leaves are killed. The experiment just described merely demonstrates that even at freezing temperatures drying of wheat leaves may take place rapidly.

It does not seem possible to differentiate between varieties which are rather similar in hardiness by merely determining their moisture contents. Under more favorable conditions for obtaining representative samples, true differences in hardiness might be shown, especially if several samples were taken and the results averaged.

#### QUANTITY OF JUICE EXPRESSED FROM LEAVES

The imbibition pressure of wheat leaves, as measured by the volume of juice or sap expressed from the leaves by given pressures, was found by Newton (34, 35, 36) to be closely related to hardiness. This test was applied in these studies during both seasons, and in 1923 on both unfrozen leaves and leaves frozen in the hardening room before pressing. In 1925 all samples were frozen before pressing.

Although measurements of expressed sap were recorded for pressures of 50, 150, 250, and 350 kgm. per square centimeter, only the quantities expressed at 350 kilograms are presented. All of these measurements, as shown by the curves in Figure 4, showed approximately the same relative values. At 50 kgm., frozen samples of Kanred and Minturki yielded, respectively, 80.6 and 79.5 per cent; at 150 kgm., 91.5 and 92 per cent; and at 250 kgm., 95.4 and 96.5 per cent of the quantity of sap expressed at 350 kgm. It was found by experiment that about 1 c. c. of sap was absorbed on the cloth, bowl, and plunger in pressing; and as the size of sample pressed varied considerably, the data for sap expressed per 100 gm. of material are corrected by adding 1 c. c. to the actual quantity of sap expressed. The pressure readings taken were in kilograms per square centimeter. A pressure of 350 kgm. per square centimeter is equal to 338.7 atmospheres, and approximately the latter measure (339 atmospheres) is used in the tables and textual discussion.

The data for the quantity of juice expressed per 100 gm. of fresh tissue at a pressure of 339 atmospheres, together with the percentage of total moisture which was expressed, are given in Tables 4 and 5 and are shown graphically in Figures 5 and 6.

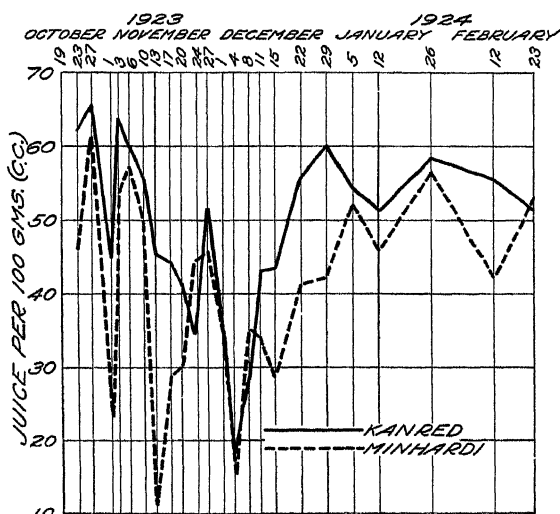


FIG. 5.—Quantities of juice expressed, at a pressure of 339 atmospheres, per 100 grams of frozen leaves of Kanred and Minhard wheats collected at intervals during the autumn and winter of 1923-24, University Farm, St. Paul, Minn.

For unfrozen leaves, the quantity of juice expressed per 100 gm. of unfrozen tissue decreased during the season from 25.4 c. c. for Minhardi and 29.1 c. c. for Kanred on October 19, to 5.2 c. c. for Minhardi and 5.3 c. c. for Kanred on December 8. After the latter date the quantity increased somewhat. A similar relation was shown in the percentages of total moisture expressed, indicating that the decreased quantity of expressed juice was not entirely a measure of

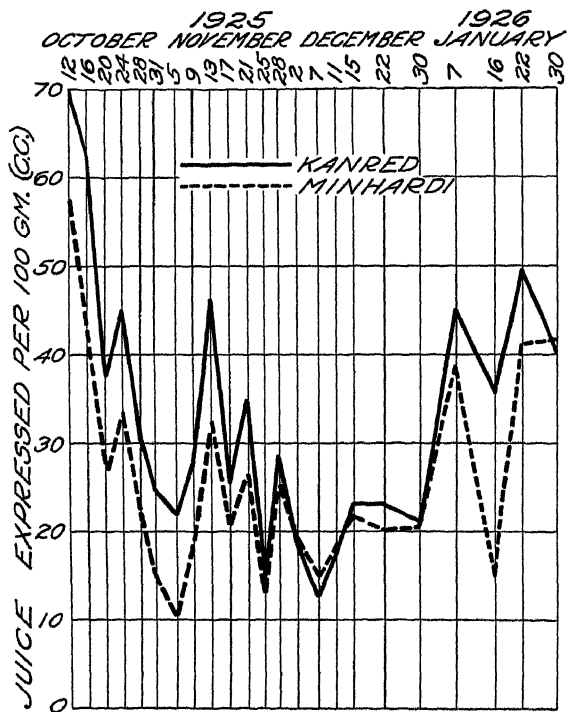


FIG. 6.—Quantities of juice expressed, at a pressure of 339 atmospheres, per 100 grams of frozen leaves of Kanred and Minhardi wheats collected at intervals during the autumn and winter of 1925-26, University Farm, St. Paul, Minn.

the decreased quantity of expressed juice was not entirely a measure of the decreased moisture content. More juice was expressed from rye and White Winter wheat than from Kanred and Minhardi wheats, because of their higher moisture content. The data in Table 4 show that considerably more juice is expressed from the frozen than from the unfrozen tissues. This is due to the increased permeability of the cell membranes and the partial precipitation of the protoplasm as a result of freezing. More juice was expressed from Kanred wheat than from Minhardi and more from White Winter than from Kanred. The quantity of juice expressed from these varieties of wheat previous to the November 20 samplings was always in the inverse order of their hardness. After that date there were some irregularities, but usually the less hardy varieties yielded more juice than Minhardi. The differences between Kanred and Minhardi in the quantity of juice expressed varied considerably, depending not only upon the moisture content but also upon the effects of freezing upon the tissues. After November 20 the differences were too irregular or too small to be of value in distinguishing between hardy and nonhardy wheats, and after December 4 the quantity of juice obtained from both varieties was too large to be used as a basis for measuring hardness.

More juice was expressed from rye than from Minhardi wheat until December 1, the date of the last collection of rye. There was little indication from the results obtained in 1923 that rye possessed greater ability to hold water against freezing and pressure than the hardy winter wheats. This may have been partly because the rye was sown nine days later than the wheat. Approximately the same

relationships were shown in percentage of total moisture expressed as in the quantities of juice expressed. The percentage expressed from White Winter wheat was more than 70 per cent except on two occasions, while Minhardi yielded less than 70 per cent of its juice most of the time, except at the beginning and end of the season.

TABLE 4.—Quantity of juice expressed, at 339 atmospheres pressure, from frozen and unfrozen plants of three varieties of wheat and one variety of rye collected at intervals during the autumn and winter of 1923-24, University Farm, St. Paul, Minn.

Condition and date collected	Juice per 100 gm. of fresh tissue (c. c.)				Percentage of total moisture expressed			
	Min-hardi	Kanred	White Winter	Minnesota No. 2 rye	Min-hardi	Kanred	White Winter	Minnesota No. 2 rye
<b>UNFROZEN PLANTS</b>								
October:								
19.....	25.4	29.1			32.6	35.9		
23.....	15.4	14.1			20.6	17.8		
27.....	16.8	20.3			24.1	28.9		
November								
1.....	13.9	16.3			20.6	22.0		
3.....	9.4	12.1	20.3	14.3	12.8	15.7	26.9	18.6
6.....	9.0	11.3			12.9	15.1		
10.....	9.9	9.5	14.6	10.1	14.1	13.1	19.9	13.4
13.....	6.1	8.5			9.0	11.9		
17.....	5.7	8.3	6.7	7.7	8.1	11.4	9.0	10.3
20.....	9.0	7.8			12.0	10.6		
24.....	7.4	8.1	8.1	11.1	10.5	12.1	11.0	16.1
27.....	6.1	5.6			9.3	7.7		
December:								
1.....	5.7	5.3	8.4	6.9	7.7	7.2	11.4	9.8
4.....	5.3	8.5			7.8	11.8		
8.....	5.2	5.3	6.0		7.6	7.6	8.7	
11 <sup>a</sup> .....	9.7	14.7			13.9	20.9		
15.....	8.4	10.0	9.2		13.5	14.8	16.5	
22.....	13.9	7.7	12.4		21.1	10.5	18.4	
29 <sup>b</sup> .....	32.4	35.5	46.5		45.8	47.8	62.2	
Average <sup>c</sup> .....	{ 10.128 ±0.810 }	{ 11.25 ±0.927 }						
<b>FROZEN PLANTS</b>								
October:								
23.....	46.0	62.1			61.7	79.0		
20.....	61.3	65.3			88.3	94.1		
November:								
1.....	23.5	44.9			33.8	61.2		
3.....	53.6	63.6	68.1	53.4	73.8	83.1	89.1	70.5
6.....	57.1	59.9			81.8	80.7		
10.....	50.0	55.4	67.1	52.3	71.2	74.5	93.3	69.9
13.....	11.3	45.4			16.6	63.9		
19.....	28.8	44.1	56.8	33.3	40.9	60.6	77.8	45.1
20.....	30.1	41.3			43.8	55.6		
24.....	44.4	34.5	51.4	50.1	65.3	50.3	70.6	71.5
27.....	45.6	51.1			67.2	70.5		
December:								
1.....	33.4	34.3	36.4	26.4	47.1	46.5	50.0	35.9
4.....	15.4	18.4			22.2	26.5		
8.....	55.0	28.2	49.1		52.6	39.3	70.9	
11.....	34.1	43.1			52.5	62.4		
15.....	28.5	43.3	35.5		43.0	63.5	66.4	
22.....	41.4	55.7	58.7		60.5	77.7	87.1	
29.....	42.2	60.0	58.8		62.4	83.6	86.4	
January:								
5.....	52.4	54.0	55.3		73.7	76.9	83.2	
12.....	45.9	51.4	50.3		64.9	75.0	73.3	
26.....	56.3	58.3	59.5		84.3	82.3	79.2	
February:								
12.....	42.0	55.3	57.1		62.2	82.6	82.5	
23.....	53.1	51.1	47.2		81.7	70.6	73.2	
March:								
8.....	41.0	37.6	27.4		73.8	59.0	42.6	
27.....	24.7	26.4			40.6	46.2		
May:								
17.....	68.2	69.4			90.8	88.2		
Average.....	{ 40.973 ±1.820 }	{ 48.235 ±1.673 }	{ 51.913 ±1.854 }					

<sup>a</sup> Samples partly frozen when gathered.

<sup>b</sup> Samples frozen when gathered.

<sup>c</sup> Not including samples collected Dec. 29.

TABLE 5.—Average quantity of juice expressed, at 339 atmospheres, from duplicate samples of frozen plants of four varieties of wheat and one variety of rye collected at intervals during the autumn and winter of 1925-26, University Farm, St. Paul, Minn.

Date collected	Juice expressed per 100 grams (c. c.)					Percentage of total moisture expressed				
	Min-hardi	Kan-red	White Winter	Mar-quis	Minnesota No. 2 rye	Min-hardi	Kan-red	White Winter	Mar-quis	Minnesota No. 2 rye
October.										
12.....	57.9	69.3	70.9	69.8	70.9	75.4	88.1	87.3	85.3	88.1
14.....	43.4	62.1	68.7	73.1	57.2	57.6	80.1	85.3	90.4	73.0
16.....	26.3	37.4	47.6	56.3	25.4	36.3	50.0	60.8	71.8	32.9
20.....	33.6	44.9	55.6	64.1	37.4	46.6	59.5	70.2	83.2	50.0
24.....	21.9	30.9	42.7	41.3	28.7	32.6	42.8	58.6	59.1	39.8
28.....	15.6	24.9	36.6	40.7	9.7	26.1	39.0	57.4	62.3	15.0
November.										
5.....	10.5	21.7	34.5	47.2	12.9	15.5	30.7	48.6	65.8	17.8
9.....	15.6	27.5	31.3	34.0	12.3	31.1	43.5	53.2	59.1	18.8
13.....	32.1	46.1	45.1	45.8	27.9	48.7	65.2	64.0	69.2	39.2
17.....	20.8	25.5	30.0	33.7	11.8	32.7	37.4	44.1	55.8	17.3
21.....	26.5	34.8	42.4	16.7	21.3	40.4	50.2	60.8	68.3	30.4
25.....	12.9	15.0	19.8	29.3	5.7	20.6	22.1	30.2	29.3	8.0
28.....	25.4	28.4	32.9	29.6	25.5	41.1	42.5	49.6	47.3	36.2
December.										
2.....	19.7	19.2	20.0	21.7	6.0	31.8	30.4	32.6	36.7	8.9
7.....	15.0	12.9	20.6	33.8	7.1	21.8	18.8	31.2	52.2	9.2
11.....	18.2	17.0	30.7	43.2	9.7	27.3	21.4	41.9	56.6	13.3
15.....	21.7	23.1	38.1	38.8	14.8	31.5	33.6	58.4	63.4	21.3
22.....	20.1	23.0	23.4	35.6	25.3	32.2	35.0	37.5	55.5	35.5
30.....	20.5	21.2	25.8	35.1	18.8	32.9	31.8	11.8	59.6	26.7
January:										
7.....	38.4	44.9	---	---	39.8	55.9	63.6	---	---	56.1
16.....	15.1	35.7	40.8	14.7	10.6	22.4	52.0	62.7	67.1	11.6
22.....	41.1	49.4	---	---	35.1	61.4	70.2	---	---	51.2
30.....	41.8	40.4	57.0	47.8	11.6	56.4	57.5	71.9	79.5	16.8
May:										
4.....	60.8	64.1	---	---	66.0	82.7	82.5	---	---	83.3
Average <sup>a</sup> {	25.961 ±1.630	32.839 ±2.030	38.929 ±2.121	43.413 ±1.907	22.848 ±2.287	38.3	46.5	55.1	62.8	31.3

<sup>a</sup> Not including samples collected May 4.

In the fall and winter of 1925-26, duplicate frozen samples of four varieties of wheat and one variety of rye were tested for the quantity of juice which could be expressed. (See Table 5.) The quantities of juice expressed from the wheat varieties were in inverse order of their hardness in most of the collections. In many of the tests, however, the quantity of expressed juice from one or more of the wheat varieties was not in the order of the hardness of those varieties. At the beginning of the season rye yielded more sap than Minhardi wheat, but after October 28 less juice was expressed from rye than from Minhardi, except on five dates. During part of this period less juice was expressed from rye than from any of the wheats. The small quantity of juice expressed from hardened tissues of Minnesota No. 2 rye, which is harder than any wheat known, shows that hardness in cereals is largely a matter of imbibition pressure of the cell colloids and tends further to substantiate the results reported by Newton (34, 35, 36).

It will be noticed that considerable variation occurs in the quantity of juice expressed, even within short periods. Large differences between collections 15 days apart, during a period in which hardening occurred, are shown in Figure 4. Some of this variation between consecutive collections is due, of course, to experimental and sampling

errors and also to differences in technic or treatment of the samples. Most of the variation, however, can be accounted for either by differences in moisture content or by the differences in the water-holding power of the tissues. This latter characteristic could be determined readily by the appearance of the samples removed from the press bowl. The hardened tissues of hardy varieties were not broken down by freezing and pressure, and when removed from the bowl they were elastic and turgid and expanded when the pressure was released; whereas samples of nonhardy or unhardened tissues remained in a thin compact layer when removed from the press bowl. This difference is shown in Figure 7. It was observed that the samples which yielded most of their juice in pressing showed the characteristics of unhardened samples mentioned above, while samples which yielded very little juice were elastic both before and after pressing. Unhardened samples when thawed were of dark color, flaccid, and watery. On several occasions the samples of Minhardi showed as much injury from freezing as Kanred or more.

The differences which exist between plants in adjoining rows or in successive periods, in the ease with which wheat leaves give up water, partly explain why winterkilling in wheat is so variable under different seasonal and environmental conditions. Adjoining rows of wheat often differ noticeably in winter survival, and winterkilling is often irregular even within a short row. Temperature conditions fatal to wheat in one season may not be injurious in another.

In the winter of 1923-24 the increased quantity of expressed juice during December coincided with a warm period in which the growth of the wheat was resumed and the hardening process reversed. The sudden drop in temperature which followed did not permit hardening to occur again before the plants were permanently frozen. The large quantity of expressed juice obtained during the winter of 1924 probably was due to the abnormal seasonal conditions just described. In December, 1925, a gradually increasing quantity of expressed juice again was obtained, this time during continued cold weather. The increased quantity of expressed juice and the apparent coming out from the hardened condition which occurred in this season might be explained, however, in one or both of two ways. Probably some of the tissues were being killed and broken down, thus losing their power of imbibition. The more probable explanation is the increased effect of gradual freezing in the breaking down of protoplasm, mentioned by Bayliss (6). The plants were continuously frozen during December but the extent of freezing was increased by subjecting the tissues to the low temperatures of the hardening room. The increased freezing probably removed additional water from the protoplasm to form ice.

It was thought that some of the variation occurring in the samples might be due to methods of handling. One of the variables was the rate and manner of thawing the material. Usually samples were taken from the freezing room to the laboratory and allowed to thaw at room temperature, but occasionally they were placed on or near the radiator to speed up the process. Slight differences were observed in the quantities of juice expressed from the tissue warmed to room temperature or slightly above, when compared with similar samples which were barely thawed.

Samples of rye were collected December 22, 1925, and were subjected to repeated freezings and thawings for different periods. The



FIG. 7.—Press residues of hardened leaves of wheat and rye plants after being frozen and pressed. Left to right: Kaured, Minhardi, and White Winter wheats, Minnesota No. 2 rye, and Marquis wheat. The hardness of the varieties is in the direct order of the fluffiness of the samples. Unhardened leaves of the hardy varieties behaved like the Marquis sample

treatments and results shown in Table 6 indicate that thawing to room temperature for a few hours and then freezing again causes only

a slight increase in the quantity of juice expressed. The sample which was frozen and thawed three times yielded only 4.1 c. c. more juice per 100 gm. than the sample thawed but once.

TABLE 6.—Average quantity of juice expressed, at 339 atmospheres, from duplicate sample plants of Minnesota No. 2 rye collected on December 22, 1925, and subjected to different thawing and freezing periods before pressing, University Farm, St. Paul, Minn.

Treatment	Date of pressing	Juice per 100 gm.
Thawed and pressed at once	Dec. 29	C. c. 25.3
Thawed 24 hours at room temperature and pressed	do	28.8
Thawed 6 hours Dec. 29, returned to hardening room, thawed 6 hours, and pressed	Jan. 2	33.9
Thawed at room temperature 6 hours Dec. 29, returned to hardening room; thawed at room temperature 6 hours Jan. 2, returned to hardening room; thawed at room temperature 5 hours and pressed	Jan. 19	29.4

TABLE 7.—Quantity of juice per gram of dry matter held, after pressing at 339 atmospheres, by frozen and unfrozen plants of three varieties of wheat and one variety of rye collected at intervals during the fall and winter of 1923-24, University Farm, St. Paul, Minn.

Date collected	Water held per gram of dry matter (grams)							
	Frozen plants				Unfrozen plants			
	Minhardi	Kanred	White Winter	Minnesota No. 2 rye	Minhardi	Kanred	White Winter	Minnesota No. 2 rye
October:								
19					2.36	2.78		
23	1.12	0.77			2.37	3.44		
27	.27	.13			1.76	1.68		
November:								
1	1.52	1.07			1.66	2.22		
3	.69	.55	0.35	0.92	2.44	2.87	2.29	2.70
6	.42	.55			1.99	2.53		
10	.68	.74	.17	.89	2.01	2.28	2.20	2.60
13	1.77	.89			1.90	2.20		
17	1.41	1.06	.60	1.55	2.22	2.39	2.60	2.63
20	1.24	1.28			2.63	2.47		
24	.74	1.09	.79	.67	2.11	1.79	2.52	1.87
27	.69	.78			1.75	2.50		
December:								
1	1.29	1.51	1.34	1.78	2.63	2.56	2.47	2.15
4	1.77	1.67			1.94	2.21		
8	.94	1.54	.66		2.01	2.16	2.02	
11	.88	.84			1.99	1.86		
15	1.12	.78	.57		1.41	1.78	1.05	
22	.85	.57	.27		1.53	2.46	1.70	
29	.78	.42	.40					
January:								
5	.65	.55	.34					
12	.84	.54	.58					
26	.36	.42	.64					
February:								
12	.80	.30	.39					
23	.34	.77	.49					
March:								
8	.34	.72	1.03					
27	.93	.72						
May:								
17	.28	.44						
Average	.874	.796			2.039	2.343		

Duplicate samples of Kanred wheat collected November 4, 1925, during a mist, were left in the laboratory for 20 hours before being placed in the hardening room. These samples had a moisture content only about 2 per cent higher than that of the samples col-

lected the following day and taken to the hardening room immediately, yet they yielded an average of 59.2 c. c. of juice per 100 gm. of tissue, as compared with 21.7 c. c. of juice per 100 gm. of tissue from the latter. Fermentation, which had begun before the first samples were taken to the hardening room, apparently had destroyed the colloidal inhibition of the protoplasm.

A determination of the juice expressed per 100 gm. of fresh tissue does not give an exact measure of the imbibitional capacity of the protoplasm, because the tissue varies in moisture content. The quantity of water held per gram of dry matter after pressing at 339 atmospheres pressure has been calculated, therefore, and the results are presented in Tables 7 and 8. With frozen samples, the placement of varieties or collections is about the same for this relationship as for the quantities of juice expressed per 100 gm. of tissue. On unfrozen samples, however, there is little or no correlation between the quantities of juice held and the hardness of the varieties. Because of their lower initial moisture content, unfrozen tissues of Minhardi wheat usually held less juice after pressing than those of Kanred.

TABLE 8.—Average quantity of juice per gram of dry matter held, after pressing at 339 atmospheres, by duplicate samples of frozen plants of four varieties of wheat and one variety of rye collected at intervals during the fall and winter of 1925-26, University Farm, St. Paul, Minn.

Date collected	Water held per gram of dry matter (grams)				
	Minhardi	Kanred	White Winter	Marquis	Minnesota No. 2 rye
October:					
12.....	0.81	0.44	0.55	0.66	0.47
16.....	1.30	.68	.61	.68	.98
20.....	1.68	1.64	1.42	1.03	2.36
24.....	1.38	1.25	.96	.56	1.49
28.....	1.40	1.48	1.12	1.14	1.56
31.....	1.07	1.09	.77	.71	1.58
November					
5.....	1.78	1.68	1.26	.88	2.18
9.....	1.02	.97	.85	.54	1.54
13.....	1.01	.85	.86	.60	1.50
17.....	1.19	1.33	1.20	.68	1.87
21.....	1.14	1.17	.91	.60	1.62
25.....	1.35	1.67	1.35	.80	2.27
28.....	1.08	1.17	.99	.88	1.51
December:					
2.....	1.11	1.25	1.13	.92	1.91
7.....	1.70	1.78	1.34	.88	2.31
11.....	1.46	1.75	1.22	.86	2.30
15.....	1.10	1.49	.78	.58	2.17
22.....	1.18	1.27	1.04	.73	1.61
30.....	1.08	1.37	.75	.57	1.76
January:					
7.....	.98	.88	-----	-----	1.07
16.....	1.61	1.07	.70	.66	2.29
22.....	.80	.70	-----	-----	1.11
30.....	1.24	1.12	.82	.32	1.88
May:					
4.....	.48	.61	-----	-----	.64
Average.....	1.206	1.196	-----	-----	1.667

#### FREEZING-POINT DEPRESSION OF EXPRESSED JUICE

The freezing-point depression of the expressed juice from the frozen plants of wheat and rye was determined in both years of the investigation. The results for 1925-26 are from duplicate samples. The data are presented in Tables 9 and 10 and are shown graphically in Figures 8 and 9.

TABLE 9.—Freezing-point depressions of the juice expressed from frozen plants of three varieties of wheat and one variety of rye collected at intervals during the fall and winter of 1923-24, University Farm, St. Paul, Minn.

Date collected	Freezing-point depression (°C.)			
	Minhardi	Kanred	White Winter	Minnesota No. 2 rye
October:				
23.....	1.11	1.18		
27.....	.87	.80		
November:				
1.....	1.57	1.47		
3.....	1.67	1.48	1.47	1.48
6.....	1.69	1.43		
10.....	1.68	1.51	1.42	1.38
13.....	.97	1.01		
17.....	1.32	1.35	1.30	1.26
20.....	1.35	1.27		
24.....	1.14	1.03	1.20	1.10
27.....	1.71	1.49		
December:				
1.....	1.60	1.30	1.34	1.20
4.....	1.42	1.32		
8.....	1.67	1.51	1.56	
11.....	2.11	2.03		
15.....	1.86	1.99	1.97	
22.....	1.34	1.25	1.18	
29.....	1.77	1.33	1.38	
January:				
5.....	1.60	1.53	1.55	
26.....	2.17	1.85	1.70	
March:				
8.....	2.16	1.26	.60	
May:				
17.....	1.30	1.15		
Average.....	{ 1.549 ±0.050 }	{ 1.388 ±0.042 }	{ 1.389 ±0.062 }	

TABLE 10.—Average freezing-point depressions of the expressed juice from plants of four varieties of wheat and one variety of rye collected at intervals during the fall and winter of 1925-26, University Farm, St. Paul, Minn.

Date collected	Freezing-point depression (°C.)				
	Minhardi	Kanred	White Winter	Marquis	Minnesota No. 2 rye
October:					
12.....	1.25	1.15	1.06	0.99	1.11
16.....	1.17	1.17	1.03	.99	1.16
24.....	1.36	1.29	1.24	1.11	1.21
28.....	1.55	1.42	1.40	1.58	1.50
31.....	2.21	2.19	2.07	2.15	2.10
November:					
5.....	.98	1.06	1.13	1.02	.91
9.....	2.13	2.14	2.01	1.83	2.17
13.....	1.58	1.43	1.46	1.54	1.37
17.....	1.70	1.72	1.76	1.96	1.71
21.....	1.57	1.44	1.51	1.58	1.42
25.....	1.69	1.62	1.86	2.12	1.41
28.....	1.90	1.80	1.90	2.17	1.77
December:					
2.....	2.05	1.89	2.03	1.90	1.66
7.....	1.14	1.18	1.36	1.47	1.13
11.....	1.21	1.20	1.29	1.03	1.19
15.....	1.39	1.36	1.36	1.15	1.37
22.....	1.99	2.03	2.06	1.19	1.55
30.....	1.88	1.85	1.93	1.33	1.74
Average (first 18 samples).....	1.597	1.552	1.581	1.506	1.471
January:					
7.....	1.45	1.43			1.29
22.....	1.47	1.33			1.29
May:					
4.....	1.11	1.04			1.08
Average a.....	{ 1.584 ±0.052 }	{ 1.535 ±0.051 }	{ 1.581 ±0.057 }	{ 1.506 ±0.066 }	{ 1.453 ±0.048 }

a Not including samples collected May 4.

The juice of the Minhardi variety had an average freezing-point depression greater than that of Kanred and White Winter in 1923-24, but about the same as White Winter in 1925-26. The juice of rye usually had a lower freezing-point depression than any of the wheats. The freezing-point depression, although fluctuating, tended to increase during the autumn, fluctuated during the winter, and then decreased in the spring after growth was well begun. The freezing-point depressions of the wheat varieties obtained during the first three collections of 1925 and also most of the early collections of 1923 were in the direct order of hardiness. Later in the season the more tender varieties were so low in moisture content that the juice was much more concentrated. The freezing-point depression of winter-wheat juice seems to depend upon the moisture content of the tissue, the quantity of soluble materials elaborated, and the quantity of imbibed water. When the plants are hardened, or partly so, and a complete and representative sample of juice can not be obtained by freezing and pressure, a freezing-point depression of the juice obtained is not a

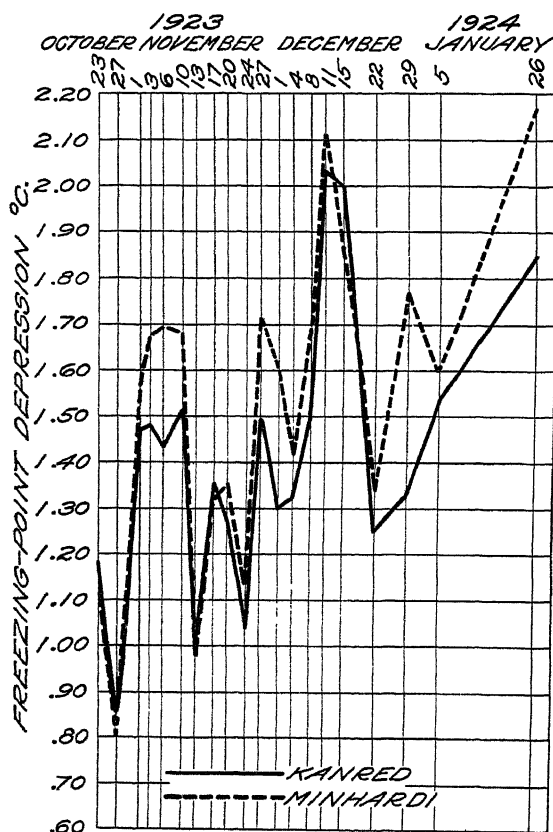


FIG. 8.—Freezing-point depression (°C.) of the juice expressed from the frozen leaves of Kanred and Minhardi wheats collected at intervals during the autumn and winter of 1923-24, University Farm, St. Paul, Minn.

measure of the hardiness of a variety of wheat. When hardening or wilting has not occurred, the freezing-point depression of the plant juice is a fairly reliable measure of hardiness in the wheat varieties studied.

#### TOTAL SOLIDS IN EXPRESSED JUICE

The percentage of total solids in the expressed juice, as measured by the Abbe refractometer, was determined from both frozen and unfrozen samples in 1923-24. The results are given in Table 11 and are shown graphically in Figure 10. Higher percentages of solids nearly always were present in the juice from frozen plants. Considerable variation existed in the juice from plants collected on different dates, due to variations both in juice concentration and in

the quantities of juice expressed. The percentage of total solids in the juice was higher in Minhardi than in Kanred, and higher in Kanred than in White Winter. The harder the variety the higher was the content of total solids in the juice on most dates and as an average. Rye juice usually had a lower content of total solids than that of any of the wheats. The total solids determined consisted of material in both molecular and colloidal solution, but probably mostly the former. The higher percentage of total solids in hardy varieties probably is due largely to the lower moisture content, and the increase in total solids during hardening both to decreased moisture content and to the increase in soluble carbohydrates and nitrogen compounds. The higher percentage of total solids in the frozen tissue shows that the disorganization of the protoplasm by freezing causes a release of soluble material not obtained by pressure without freezing. The juice obtained from the unfrozen tissue probably came largely from the vacuoles.

TABLE 11.—Total solids in the juice, expressed at 339 atmospheres pressure, from unfrozen and frozen plants of three varieties of wheat and one variety of rye collected at intervals during the fall and winter of 1923-24, University Farm, St. Paul, Minn.

Date collected	Total solids (per cent)							
	Unfrozen samples				Frozen samples			
	Min-hardi	Kanred	White Winter	Minnesota No. 2 rye	Min-hardi	Kanred	White winter	Minnesota No. 2 rye
October:								
19.....	8.8	9.3	-----	-----	11.2	10.0	-----	-----
23.....	13.6	11.6	-----	-----	-----	-----	-----	-----
27*.....	3.6	2.7	-----	-----	9.0	7.0	-----	-----
November:								
1.....	13.5	8.0	-----	-----	18.0	17.5	-----	-----
3.....	13.0	11.0	9.0	10.0	15.0	13.8	12.3	13.5
6.....	10.5	8.5	-----	-----	16.5	14.0	-----	-----
10.....	10.5	10.5	11.0	11.0	16.5	14.3	13.8	13.5
13.....	9.5	10.0	-----	-----	11.0	10.5	-----	-----
17.....	12.0	10.4	12.5	10.0	12.5	12.3	11.2	11.3
20.....	11.0	11.0	-----	-----	14.5	13.3	-----	-----
24.....	8.0	9.5	8.3	8.0	12.0	10.0	11.0	11.0
27.....	9.5	10.5	-----	-----	16.5	14.5	-----	-----
December:								
1.....	9.8	7.0	7.5	5.4	13.0	10.0	10.0	8.0
4.....	10.0	10.0	-----	-----	10.5	9.0	-----	-----
8.....	12.7	11.3	10.0	-----	18.0	14.0	15.0	-----
11.....	15.5	13.0	-----	-----	19.5	18.5	-----	-----
15.....	19.5	16.5	15.0	-----	18.0	20.0	18.0	-----
22.....	14.0	14.0	8.0	-----	13.0	12.0	11.5	-----
29.....	-----	-----	-----	-----	15.5	10.0	13.0	-----
January:								
5.....	-----	-----	-----	-----	12.3	11.0	12.1	-----
12.....	-----	-----	-----	-----	14.8	11.0	10.5	-----
26.....	-----	-----	-----	-----	16.5	11.8	11.6	-----
February:								
12.....	-----	-----	-----	-----	12.5	12.9	6.5	-----
26.....	-----	-----	-----	-----	10.7	8.5	11.5	-----
March:								
8.....	-----	-----	-----	-----	18.0	10.0	6.0	-----
27.....	-----	-----	-----	-----	11.0	9.5	-----	-----
May:								
17.....	-----	-----	-----	-----	4.2	6.3	-----	-----
Average.....	{ 11.389 ±0.524 }	{ 10.267 ±0.447 }	-----	-----	{ 13.854 ±0.460 }	{ 11.988 ±0.428 }	{ 11.600 ±0.496 }	-----

\* Samples gathered on Oct. 27 contained excess moisture due to snow.

## BOUND WATER IN EXPRESSED JUICE

The determination of bound water was made in 1923-24 on juice from frozen samples only. The average percentage of bound water in the juice of Minhardi, Kanred, and White Winter varieties varied directly with hardness. Rye juice showed a bound-water content greater than that of wheat on some dates and less on others. There was extreme irregularity in the quantities of bound water present. The quantity depends both on the binding power of the colloids in the cells and on the quantity of colloidal material which is removed in expressing the juice. Probably the effect of freezing on the precipitation and dispersion of the colloids also has some effect. The plant tissue was not macerated before pressing, so it is unlikely that the colloids in the juice represent the colloidal conditions within the cells. The fluctuations in the percentage of bound water in the juice obtained by the method used render this test of little value in measuring hardness. The data on bound water are given in Table 12 and are shown graphically in Figure 11.

TABLE 12.—Bound water in the juice expressed from the frozen plants of three varieties of wheat and one variety of rye collected at intervals during the autumn and winter of 1923-24, University Farm, St. Paul, Minn.

Date collected	Bound water (per cent)			
	Minhardi	Kanred	White Winter	Minnesota No. 2 rye
October.				
23.....	5.8	7.8		
27.....	6.5	10.0		
November.				
1.....	0	1.1		
3.....			1.9	
10.....	9.7	10.1	8.0	13.9
13.....		4.7		
17.....	16.6	7.3	11.1	5.0
20.....	15.4	13.3		
24.....	8.3	4.3	2.7	8.0
27.....	6.5	8.0		
December:				
1.....	4.7	12.3	5.0	10.4
4.....	7.3	7.3		
8.....	17.1	9.4	6.9	
11.....	14.2	5.0		
15.....	3.5	1.5	6.9	
22.....	3.5	2.3	5.0	
29.....	8.0	10.4	6.9	
January:				
5.....	3.9	9.7	6.5	
26.....	7.3	8.0		
March:				
8.....	16.3	9.0		
May:				
17.....	11.9	11.6		
Average.....	{ 8.763 ±0.785 }	{ 7.655 ±0.516 }	{ 6.090 ±0.532 }	}

## CORRELATIONS BETWEEN CHARACTERS USED TO MEASURE HARDNESS

In order to determine the relation of the results obtained in the various tests on the wheat and rye samples, correlation coefficients were calculated. The variables used were from the average of duplicate determinations made in 1925-26 and from single determinations made in 1923-24, except that the moisture-content determinations of

this season also were in duplicate. Data obtained during the entire season were used, thus including data from both hardened and non-hardened plants. The data used in calculating the coefficients were from 15 to 26 experiments in each case, except those on White Winter wheat in 1923-24, of which only 8 to 12 items were available. The correlation coefficients were computed according to the usual formula when using ungrouped variables:

$$r = \frac{\frac{\sum xy}{n} - \left(\frac{\sum x}{n}\right) \left(\frac{\sum y}{n}\right)}{\sqrt{\frac{\sum x^2}{n} - \left(\frac{\sum x}{n}\right)^2} \times \sqrt{\frac{\sum y^2}{n} - \left(\frac{\sum y}{n}\right)^2}}$$

when  $\Sigma$  = summation,  $x$  = values of one variable,  $y$  = values of the other variable, and  $n$  = number of observations.

The correlation coefficients obtained are given in Table 13. The quantity of juice expressed is positively correlated, in most cases highly so, with the moisture content of both frozen and unfrozen plants. It is negatively correlated in most cases with freezing-point depression and percentage of totalsolids in the juice. The freezing-point depression shows a high positive correlation with the percentage of total solids and, especially in 1925-26, a high negative correlation with moisture content. The percentage of total solids is negatively correlated with moisture content. The percentage of bound water does not show any consistent correlations with the other factors measured, the coefficients with Kanred even differing in sign from those of the other two wheat varieties.

The quantity of juice expressed per 100 gm. of tissue is not an entirely comparable measure when the varieties differ in moisture content. Correlations between the quantity of juice held against freezing and pressure and the moisture content were calculated, therefore, and the coefficients shown in Table 14 were obtained.

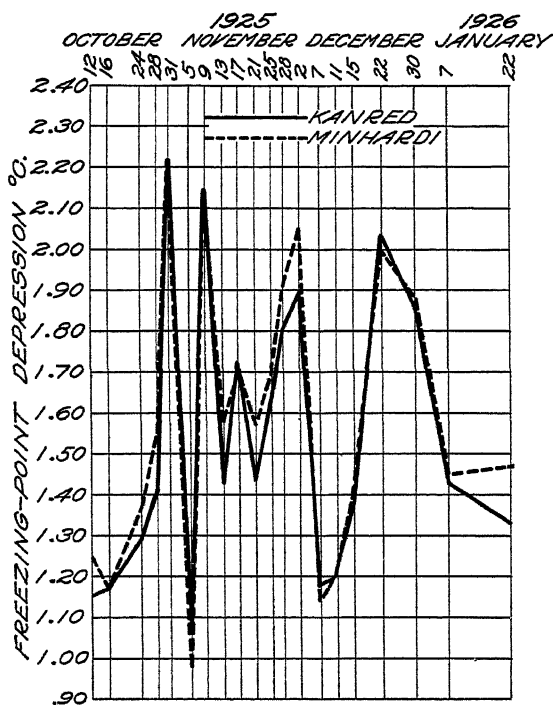


FIG. 9.—Freezing-point depression (°C.) of the juice expressed from the frozen leaves of Kanred and Minhardi wheats collected at interval, during the autumn and winter of 1925-26, University Farm, St. Pauls Minn.

TABLE 13.—*Correlation coefficients between physical factors of plants of four varieties of wheat and one variety of rye collected at intervals during the autumns and winters of 1923-24 and 1925-26, University Farm, St. Paul, Minn.*

Factor and variety	Correlation coefficients (r) between—			
	Juice expressed (cubic centimeters per 100 gm.)	Freezing point depression °C.	Percentage of total solids	Percentage of bound water
<i>Frozen samples, 1923-24</i>				
Moisture content (per cent):				
Minhardi.....	+0.313±0.119	-0.462±0.113	-0.351±0.116	-0.168±0.150
Kanred.....	+ .533± .095	- .304± .131	- .008± .132	+ .274± .140
White Winter.....	+ .654± .100	- .094± .193	- .205± .167	- .192± .205
Juice expressed (cubic centimeters per 100 grams):				
Minhardi.....		+ .079± .143	- .204± .127	- .017± .155
Kanred.....		- .102± .142	- .107± .131	+ .151± .147
White Winter.....		- .405± .163	+ .169± .169	- .045± .213
Freezing-point depression (°C.):				
Minhardi.....			+ .754± .062	+ .166± .150
Kanred.....			+ .782± .056	- .276± .139
White Winter.....			+ .873± .046	+ .160± .208
Total solids (per cent):				
Minhardi.....				+ .094± .153
Kanred.....				- .533± .108
White Winter.....				+ .225± .202
<i>Unfrozen samples, 1923-24</i>				
Moisture content (per cent):				
Minhardi.....	+ .561± .109		- .336± .141	
Kanred.....	+ .477± .123		- .110± .156	
White Winter.....	+ .280± .220		- .634± .143	
Juice expressed (cubic centimeters per 100 grams):				
Minhardi.....			- .160± .155	
Kanred.....			- .312± .144	
White Winter.....			- .191± .230	
<i>Frozen samples, 1925-26</i>				
Moisture content (per cent):				
Rye.....	+ .717± .068	- .757± .064		
Minhardi.....	+ .696± .073	- .827± .049		
Kanred.....	+ .734± .065	- .805± .053		
White Winter.....	+ .868± .036	- .674± .087		
Marquis.....	+ .897± .029	- .624± .097		
Juice expressed (cubic centimeters per 100 grams):				
Rye.....		- .388± .128		
Minhardi.....		- .310± .136		
Kanred.....		- .387± .128		
White Winter.....		- .811± .054		
Marquis.....		- .670± .088		

TABLE 14.—*Calculated correlation coefficients between the quantity of juice held against freezing and pressure and moisture content of plants of Kanred and Minhardi wheats*

Variety	Correlation coefficients (r)	
	Frozen samples	Unfrozen samples
Kanred.....	0.088±0.131	0.898±0.030
Minhardi.....	.112± .131	.916± .026

While the correlation between moisture content and juice held against expression is positive, it is too small in the frozen samples to be significant. With the unfrozen samples, on the other hand, the correlation coefficients of 0.898 and 0.916 indicate that the moisture content probably is the chief factor in determining how much juice can be expressed from the tissues. If the tissues are sufficiently wilted, no juice can be expressed from the samples at the pressures used.

The correlations between the quantity of juice expressed and the moisture content of the frozen samples in 1925-26 are much higher

than in 1923-24. It will be observed that the values of the correlation coefficients in the wheats in both seasons are in the inverse order of hardiness. The regression coefficients for the samples of 1925-26 were calculated, and the regression lines for the moisture contents for given quantities of juice expressed are shown in Figure 12. By extrapolating the regression lines somewhat beyond the limits of the data, the approximate moisture content at which no juice could be expressed at 339 atmospheres pressure after freezing is shown. These theoretical moisture contents at

which sap can not be expressed are in the direct order of the hardiness of the varieties, except that Kanred and Minhardi are reversed. This shift is the result partly of an occasional larger juice yield from Minhardi than from Kanred late in the season, but chiefly of the lower average moisture content of Minhardi and its ability to thrive at a considerably lower moisture content.

Partial correlation coefficients were calculated on some of the factors, to compensate for the variation in the moisture content of the samples. These coefficients are given in Table 15. The partial correlation coefficients between juice expressed per 100 gm. of tissue and freezing-point depression are not significantly different from the gross correlation coefficients for 1923-24. Slight positive but not significant partial correlations between juice expressed and bound water were obtained in all three varieties; the higher the correlation the less hardy is the variety. It is still apparent that bound water in

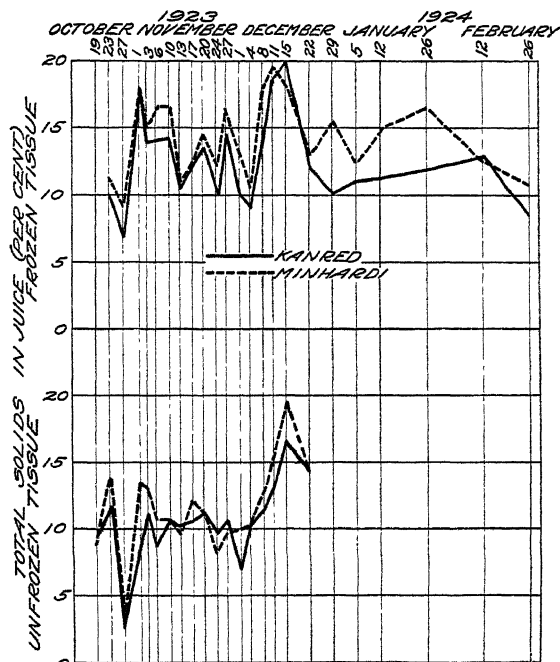


Fig. 10.—Percentage of total solids in the juice expressed from frozen and unfrozen leaves of Kanred and Minhardi wheats collected at intervals during the autumn and winter of 1923-24, at University Farm, St. Paul, Minn.

the juice expressed from unground frozen tissue has little direct relation to imbibition pressure in the tissues, but the trend of the partial correlation coefficients indicates that the more the tissue is broken down in freezing and pressing the more colloidal material the juice will contain.

TABLE 15.—The gross correlation coefficients for juice expressed per 100 gm. of tissue with freezing-point depression and bound water and the partial correlation coefficients (assuming that the moisture content is held constant) obtained on wheat and rye in 1923-24 and 1925-26

Season and variety	Correlation coefficients for juice expressed per 100 grams of tissue			
	Gross correlation coefficients		Partial correlation coefficients <sup>a</sup> (moisture content constant)	
	Freezing-point depression ( <i>r</i> <sub>23</sub> )	Bound water ( <i>r</i> <sub>24</sub> )	Freezing-point depression ( <i>r</i> <sub>23.1</sub> )	Bound water ( <i>r</i> <sub>24.1</sub> )
Season of 1923-24				
Minhardi.....	+0.079	−0.017	+0.265	+0.004
Kanred.....	−.102	+ .151	+ .198	+ .001
White Winter.....	−.405	−.045	−.456	+ .109
Season of 1925-26:				
Rye.....	−.388	.....	.....	+ .340
Minhardi.....	−.310	.....	.....	+ .641
Kanred.....	−.387	.....	.....	+ .506
White Winter.....	−.811	.....	.....	−.616
Marquis.....	−.670	.....	.....	−.319

<sup>a</sup> Partial correlation coefficients were computed by the formula:  $r_{23.1} = \frac{r_{23} - (r_{12} \times r_{13})}{\sqrt{1-r_{12}^2} \sqrt{1-r_{13}^2}}$

The nonhardy varieties, Marquis and White Winter, had much higher gross correlations between juice expressed and freezing-point depression than the more hardy varieties, Minhardi, Kanred, and Minnesota No. 2 rye. This showed that the quantity of juice expressed from the less

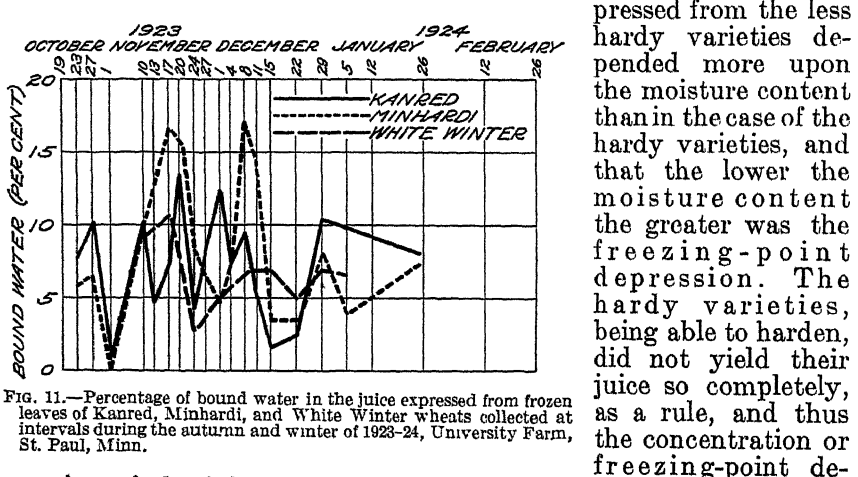


FIG. 11.—Percentage of bound water in the juice expressed from frozen leaves of Kanred, Minhardi, and White Winter wheats collected at intervals during the autumn and winter of 1923-24, University Farm, St. Paul, Minn.

hardy varieties depended more upon the moisture content than in the case of the hardy varieties, and that the lower the moisture content the greater was the freezing-point depression. The hardy varieties, being able to harden, did not yield their juice so completely, as a rule, and thus the concentration or freezing-point depression of the juice varied both with the moisture content of the tissue and in the extent to which the tissue was broken down in freezing and pressing. The partial correlations, assuming moisture content to be constant, still show the freezing-point depression of the juice of the two nonhardy varieties, Marquis and White Winter, to

be negatively correlated with the quantity of juice expressed, while in the juice of the three hardy varieties, Minhardi, Kanred, and Minnesota No. 2 rye, these two factors are positively correlated. These results may be merely chance differences, as such relationships were not obtained in the 1923-24 samples. On the other hand, they may indicate that hardy varieties usually yield the more dilute vacuolar sap, but as the protoplasm is broken down, juice which contains more dissolved material is expressed. The nonhardy varieties are almost completely broken down by the freezing at all times, and it appears that the more completely the juice is expressed the more dilute it becomes.

#### RATE OF RESPIRATION AT LOW TEMPERATURES

The results obtained by Govorov (18) indicated that hardy wheats and ryes respired less than nonhardy varieties at low temperatures.

Newton (36) found that hardy varieties retained their sugars throughout the winter more completely than the nonhardy varieties. Experiments were undertaken by the writer to determine whether these sugars were used up as the result of a greater rate of respiration. Plants of wheat and rye were grown in glazed pots in the greenhouse. Before starting a pot of plants in the respiration experiment,

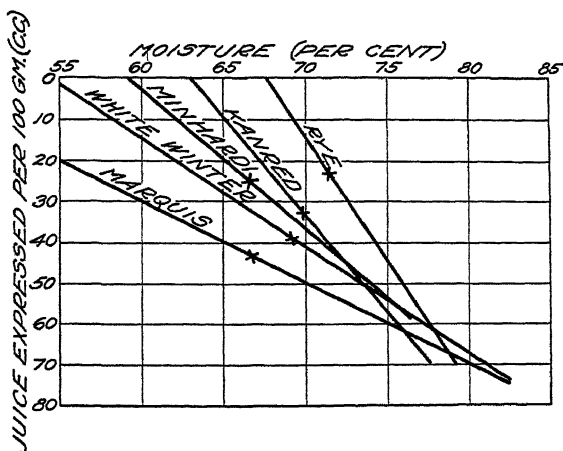


FIG. 12.—Regression lines for the mean values of moisture content plotted against the quantity of juice expressed per 100 gm. of frozen leaves of four varieties of wheat and one variety of rye

the soil was sealed over with a half-and-half mixture of vaseline and liquid paraffin, which, after melting, could be poured at a temperature of 40° to 45° C. on the soil and around the bases of the plants. The cover was then placed over the plants with its edge resting on the seal inside the pot. The space between the cover and the pot then was sealed. The pots were first placed in the chamber at 5° C., and when the rate of respiration had been determined they were transferred successively to other chambers with temperatures of 0°, -5°, and -10° C., respectively. After the pots had come to a temperature equilibrium in a chamber, 8 to 12 hours being allowed, the CO<sub>2</sub> present was removed with a stream of air from which the CO<sub>2</sub> had been removed, and the plants were allowed to respire. The gradual reduction in temperature permitted the winter varieties to survive the final temperature of -10° C., but the spring wheat Marquis did not survive. The winter varieties showed growth after passing through the respiration experiments and being cut off just above the wax seal. This indicated that the seal used was not toxic. The data obtained in the respiration experiment are presented in Table 16.

TABLE 16.—Quantity of CO<sub>2</sub> respired by plants of four varieties of wheat and one variety of rye placed successively in chambers at four different temperatures

Variety and sample	Milligrams of CO <sub>2</sub> respired per hour per kilogram of dry matter at—				Van't-Hoff coefficient —10° to 0° C.
	5° C.	0° C.	—5° C.	—10° C.	
Minhardi.....	126.4	313.0	221.2	66.3	4.7
.....	320.5	142.2	163.0	60.8	2.3
.....	435.0	322.6	120.9	65.7	4.9
Average.....	294.0	259.3	168.4	64.3	4.0
Kanred.....	351.5	299.5	141.4	88.3	3.5
.....	323.9	270.6	243.3	185.5	1.4
.....	258.7	200.2	181.2	92.1	2.2
Average.....	311.4	256.8	188.6	122.0	2.4
Red Rock.....	473.5	306.7	219.3	217.1	1.4
.....	150.7	37.7	31.3	21.9	1.7
.....	357.4	203.2	173.4	49.8	4.1
Average.....	327.2	182.5	141.3	96.3	2.4
Marquis.....	514.9	261.6	143.3	53.0	4.9
.....	475.0	316.5	225.0	53.9	5.9
.....	325.5	143.0	193.8	44.0	3.3
Average.....	438.5	240.4	187.4	50.3	4.7
Rye.....	672.7	689.6	106.5	30.1	22.9
.....	359.5	433.0	158.5	58.2	7.4
.....	442.8	428.2	232.4	124.0	3.5
Average.....	491.7	516.9	165.8	70.8	11.3

At the higher temperatures, 5° and 0° C., rye showed the greatest rate of respiration. Marquis, a spring wheat, showed the second greatest rate of respiration at 5°. The low respiration of Minhardi in the first experiment at 5° C. can not be explained. A plant adjustment may have been taking place, or possibly the temperature or air-circulation controls were temporarily out of order and this was not detected. The latter possibility does not seem likely, however, as the quantity of CO<sub>2</sub> respired by the Minhardi plants at 5° C. agreed very closely during two successive periods, indicating an actual depression in respiration.

At the low temperature of —10° C. the quantity of CO<sub>2</sub> obtained per kilogram of dry matter in the winter varieties was in the inverse order of hardiness. The low rate of respiration of Marquis at —10° C. probably was due to the plants having been killed while the winter wheats and rye were not. The Van't-Hoff coefficients calculated for the range of —10° C. to 0° C. show that the increase in respiration between these temperatures was in the order of hardiness of the winter grains. The results, especially those with rye, indicate that hardy varieties are able to conserve their carbohydrates at low temperatures to a much greater extent than are the nonhardy.

#### DETERMINATION OF LETHAL TEMPERATURES FOR WHEAT

The accidental killing of two of the four varieties of wheat growing in pots in the greenhouse when they were placed outside to harden showed that varieties differed in their ability to withstand low temperatures without previous hardening. Experiments were then begun

to determine the lethal temperatures of wheat and rye taken directly from the greenhouse and also to determine the protection afforded by previous exposure to different low temperatures before subjecting them to temperatures likely to kill. The results obtained are shown in Table 17.

The tests in 1924 were made in the large refrigeration chamber belonging to the division of entomology of the University of Minnesota, and those in 1926 were made in the ice-cream cabinet used for the respiration experiments.

TABLE 17.—*Rye and wheat plants killed by exposure to various temperatures during various periods in 1924 and 1926*

Temperatures (° C.) and duration of exposure	Plants killed (per cent)									
	Minnesota No. 2 rye	Minhardi	Kanred	Fulcaster	Dawson	Red Rock	White Winter	Marquis	Kubanka	Hard Federation Kanred×Minhardi, F <sub>2</sub> hybrids
RESULTS IN 1924										
−4° 24 hours								0		
−8° 24 hours	0	0	0				96	100		
−10° 24 hours	100	100	100				100			
−12° 24 hours		100	100							
−12° to −15° 24 hours	100	100	100							
−15° 24 hours	100	100	100							
1° 72 hours, −8° 36 hours, −12° 12 hours		50	100							55.3
1° 72 hours, −8° 36 hours, −9° 12 hours		18	6				100			62.6
3° to 6° one month, −10° 24 hours							100			
3° to 6° one month, −15° 24 hours	100	100	100							
RESULTS IN 1926										
−10° 48 hours	100					100				
−7° 24 hours	0		0	50		0	100	100	100	100
−5° 24 hours			5				0	100	100	
−5° 24 hours (old plants)							0	0	0	
−4° 24 hours			0							0
5° 48 hours, −10° 24 hours		50								
0° 48 hours, −10° 24 hours		20								
−5° 48 hours, −10° 24 hours		80								
0° 48 hours, −5° 24 hours, −7° 48 hours	20	0	39	100	100					
−7° 24 hours (wilted plants)	0		0							
−7° 24 hours (not wilted)	100		100							

All varieties of wheat and rye tested were killed by an exposure for 24 hours to temperatures of −10° C. or lower after being taken directly from the greenhouse. The Minnesota No. 2 rye and the Minhardi and Kanred wheats completely survived temperatures of −8° C. for 24 hours, while 96 per cent of the White Winter and all of the Marquis plants were killed. At −7° C. for 24 hours the rye, Kanred wheat, and one pot of Red Rock wheat survived completely. One lot of Fulcaster was completely killed at the −7° C. temperature, but another lot completely survived although showing severe injury. White Winter, Marquis, and Kubanka were completely killed at −7° C. At −5° C., Kubanka and Hard Federation spring wheats were completely killed. Two lots of Marquis also were

completely killed at this temperature, although one pot containing older plants survived completely at this temperature. White Winter completely survived a temperature of  $-5^{\circ}\text{C}.$ , as also did all lots of Kanred but one, which showed some injury. Plants of Marquis, which were starting to head, completely survived a temperature of  $-4^{\circ}\text{C}.$  for 24 hours. Plants of Marquis, Hard Federation, Kubanka, White Winter, and Kanred, which had not quite reached the jointing stage, completely survived a temperature of  $-4^{\circ}\text{C}.$  for 24 hours.

In tests with Minhardi wheat at  $0^{\circ}\text{C}.$  for 48 hours, 80 per cent survived, while at  $-5^{\circ}\text{C}.$  for 48 hours only 20 per cent of the plants survived. A previous exposure to  $5^{\circ}\text{C}.$  for 48 hours enabled half of the plants to survive  $-10^{\circ}\text{C}.$  for 24 hours. In tests in which the temperatures successively were  $0^{\circ}\text{C}.$  for 48 hours,  $-5^{\circ}\text{C}.$  for 24 hours, and  $-7^{\circ}\text{C}.$  for 48 hours, 20 per cent of the rye, 39 per cent of the Kanred, and all of the Fulcaster and Dawson, but none of the Minhardi plants, were killed. In other tests, in which the temperatures used were  $1^{\circ}\text{C}.$  for 72 hours,  $-8^{\circ}\text{C}.$  for 36 hours, and  $-12^{\circ}\text{C}.$  for 12 hours, half of the Minhardi and all of the Kanred plants were killed, while with temperatures of  $1^{\circ}\text{C}.$  for 72 hours,  $-8^{\circ}\text{C}.$  for 36 hours, and  $-9^{\circ}\text{C}.$  for 12 hours, 18 per cent of the Minhardi plants, 6 per cent of the Kanred plants, and all of the White Winter plants were killed. Plants of White Winter kept for about a month at  $3^{\circ}$  to  $6^{\circ}\text{C}.$  could not withstand  $-10^{\circ}\text{C}.$  for 24 hours, nor could rye, Minhardi, and Kanred, kept under the same conditions, survive  $-15^{\circ}\text{C}.$  for 24 hours.

No consistent differences could be observed in the hardiness of wheat seedlings grown in the greenhouse and varying in age from 6 weeks to 4 months.

Plants of Minnesota No. 2 rye and Kanred wheat, which were severely wilted owing to a lack of soil moisture, recovered completely from a freezing of  $-7^{\circ}\text{C}.$ , while unwilted plants of the same varieties liberally watered were completely killed by this temperature.

#### HARDINESS OF PLANT PARTS

Plant parts differ in their ability to withstand freezing or desiccation, as shown by Schander and Schaffnit (50), Schaffnit (48), and Chandler (9). When wheat plants are frozen the tips of the oldest leaves show the first injury and wilting, but at extremely low temperatures the entire leaf is wilted. Injury progresses in a given plant from the older to the younger leaves, upon exposure to successive intensities of cold. The same effect occurs in a series of plants each held at one of a series of constant low temperatures. The crown containing the meristematic tissues is the most hardy part of the wheat plant, at least of the portion above the soil surface. Under severe winter conditions all of the wheat leaves die, but if the crown is alive recovery takes place.

In order to determine whether or not all parts of the plants had similar physicochemical properties, different portions of the plants were tested separately on one date in 1923 for moisture content, expressed juice, total solids in the juice, freezing-point depression of the juice, and bound water in the juice, and on two dates in 1925 for moisture content, expressed juice, and freezing-point depression. The results of these determinations are given in Table 18.

TABLE 18.—*Moisture content, juice expressed, total solids in the juice, freezing-point depression of the juice, and bound water in the juice obtained from plant parts of wheat and rye in 1923 and 1925, University Farm, St. Paul, Minn.*

Variety, date of collection, and plant parts	Moisture	Juice expressed per 100 grams at 339 atmospheres	Total solids in juice	Freezing-point depression	Bound water
Minhardi wheat, Dec. 1, 1923.	<i>Per cent</i>	<i>C c.</i>	<i>Per cent</i>	<i>° C.</i>	<i>Per cent</i>
Leaves, unfrozen.....	74.0	5 7	9.8	-----	-----
Roots and crown, unfrozen.....	67.2	13.4	6.5	-----	-----
Leaves, frozen.....	70.9	33.4	13.0	1.60	4.7
Roots and crown, frozen.....	65.1	29.9	11.0	1.48	15.7
White Winter wheat, Dec. 1, 1923					
Leaves, unfrozen.....	73.6	11.4	7.5	-----	-----
Roots and crown, unfrozen.....	67.8	21.1	6.0	-----	-----
Leaves, frozen.....	72.8	50.0	10.0	1.34	5.0
Roots and crown, frozen.....	68.7	37.0	9.0	1.09	12.8
Kanred wheat, Nov. 5, 1925:					
Crown, frozen.....	70.9	53.5	-----	1.56	-----
Young leaves, frozen.....	72.9	55.9	-----	1.59	-----
Old leaves, frozen.....	71.3	59.4	-----	1.52	-----
Minn. No. 2 rye, Nov. 18, 1925.					
Roots, frozen.....	79.3	55.0	-----	.58	-----
Crown, frozen.....	70.6	26.2	-----	1.44	-----
Young leaves, frozen.....	69.8	45.7	-----	1.50	-----
Old leaves, frozen.....	70.0	45.3	-----	1.54	-----

<sup>a</sup> Some wash water on roots, which accounts for higher moisture content, more juice expressed, and lower freezing-point depression.

The frozen samples of the crown and roots of Minhardi and White Winter wheats collected December 1, 1923, contained slightly less moisture than the leaves and yielded correspondingly less juice. The total solids were about the same, and the freezing-point depression of the juice from the crown and roots was about the same as those of the leaves. The chief difference in the two plant portions was in the percentage of bound water. More than three times as much bound water was found in the juice of the crowns and roots as in the leaves of Minhardi, and nearly two and one half times as much in the case of White Winter. Tests for moisture content, quantity of juice expressed, and freezing-point depression, made on the crowns, young leaves, and old leaves of Kanred wheat collected November 5, 1925, yielded very similar results. None of the tissues showed any indication of being especially hardened at this time, although Kanred leaves collected in the forenoon of the same day and left in the laboratory a shorter time yielded less than half as much juice. This indicates that hardening takes place throughout the living portion of the plant and that changes can take place very quickly. The samples of rye collected on November 18, 1925, showed that the crown yielded less juice and consequently was the hardest portion of the plant. Accurate determinations could not be made on the roots because of the necessity of washing the dirt from the roots with water.

## HARDINESS IN WHEAT HYBRIDS

### FIELD EXPERIMENTS

The inheritance of hardiness in wheat has been studied by Nilsson-Ehle (39) and by Hayes and Garber (20). Hybrids were obtained which were harder than either parent, but the genetic factors involved in hardiness were not determined. Gaines, according to

Schafer (47),<sup>6</sup> reported a recessiveness of hardiness in crosses between Turkey (winter) and Jenkin (spring Club) wheats.

The survival of some winter-wheat hybrids in comparison with the parent varieties, the protection of winter wheat obtained by sowing in stubble, and some of the difficulties encountered in determining the hardiness of hybrids in the field will now be considered. Most of these data were obtained in connection with breeding experiments of the western wheat investigations project of the Office of Cereal Crops and Diseases, which were carried on cooperatively at several western field stations. Some results, however, were secured at University Farm, St. Paul, Minn.

The bulked progenies of 36 different crosses, involving combinations of 16 hardy winter-wheat varieties, were grown in the  $F_3$  generation at Dickinson, N. Dak., in 1922 on both fallow and stubble land. Most of the wheat hybrids and parent varieties sown on fallow land were completely winterkilled, and only six crosses showed more than 1 per cent survival. On stubble land the survival of the hybrids ranged from 3 to 100 per cent. The hybrids were mostly intermediate between the parents in their survival, but a few showed survivals approaching the less hardy or the more hardy parent.

In the field the survival of three crosses was observed in detail in the  $F_3$  generation from the progenies of  $F_2$  plants. The cross Kanred  $\times$  Minhardi was grown at Dickinson, N. Dak., in 1923 and at St. Paul, Minn., in 1926; the Buffum  $\times$  Eureka cross was grown at Fargo, N. Dak., in 1922 and at Moccasin, Mont., in 1923; and the Minturki  $\times$  Turkey cross was grown at Dickinson, N. Dak., in 1923. Attempts to study several other crosses were prevented because of either complete killing or of no killing. The number of hybrid lines varied from 99 to 257 in the different tests, in comparison with 11 to 50 parental rows. The hybrid material was all grown under conditions free from winterkilling in the  $F_1$  and  $F_2$  generations. The Kanred  $\times$  Minhardi hybrids showed survivals approximately intermediate between the parents. The Buffum  $\times$  Eureka hybrids showed a survival similar to that of the more hardy Buffum parent under the mild conditions at Fargo, N. Dak., in 1922, but under more severe conditions at Moccasin, Mont., in 1923, they were killed as badly as the less hardy Eureka parent.

The Minturki  $\times$  Turkey hybrid lines survived about as well as the average of the parent varieties. The genetic factors for winter hardiness could not be determined in any of the crosses studied. Much difficulty was encountered in attempting to determine the survival of hybrids in the field, because of the irregularity of winter-killing and the frequency of complete survival or complete killing in several localities.

#### ARTIFICIAL FREEZING EXPERIMENTS

Artificial freezing was attempted at University Farm, St. Paul, Minn., in order to determine the inheritance of hardiness. Plants were grown in pots in the greenhouse, subjected to low temperatures in a refrigerator, and then returned to the greenhouse and their recovery noted. The results are shown in Table 17.

<sup>6</sup> These inheritance studies were made by E. F. Gaines, cerealist of the Washington Agricultural Experiment Station, in cooperation with the Office of Cereal Crops and Diseases of the United States Department of Agriculture.

In the test involving the series of temperatures, 1° C. for 72 hours, -8° C. for 36 hours, and -12° C. for 12 hours, in which all of the Kanred plants and half of the Minhardi plants were killed, 26 out of 47 or 55.3 per cent, of the F<sub>2</sub> hybrid plants of the cross between these two varieties were killed. This indicated a dominance of hardiness. In the other experiment, under somewhat less severe conditions with successive temperatures of 1° C. for 72 hours, -8° C. for 36 hours, and -9° C. for 12 hours, 62.6 per cent of a total population of 369 F<sub>2</sub> plants were killed. In this test, however, Minhardi was injured somewhat more than Kanred, so the significance of this survival could not be determined.

The only facts regarding the nature of inheritance of hardiness brought out by all of these results are that most of the F<sub>3</sub> strains tend to be intermediate to the parents in hardiness and that the hardier the parents used in the cross are the greater will be the average hardiness of the hybrids. The F<sub>3</sub> and later progenies may show all degrees of survival varying from that of one parent to that of the other, with possibly some transgressive inheritance, as indicated by strains being more or less hardy than either parent. Hardiness in wheat varieties seems to differ over a wide and continuous range. Many varieties doubtless differ genetically in one or more of the factors determining hardiness. It is difficult to find conditions under which one of two varieties of wheat of the same class kills entirely and the other completely survives. Usually both varieties are partly killed, or completely killed, or both survive completely. The extent to which the parents are killed seems largely to determine the extent of killing in their hybrid progeny.

#### MOISTURE CONTENT OF HYBRIDS

After observing that the difference in moisture content between Kanred and Minhardi wheats existed under nearly all conditions and that moisture content appeared to be related to winter hardiness, it seemed feasible to determine how the moisture difference was inherited.

The material selected for this experiment consisted of the progeny from each of 152 F<sub>2</sub> plants from a Kanred × Minhardi cross grown at University Farm in 1924 in 5-foot rows in conjunction with the parent varieties which occupied adjoining rows distributed at intervals of every eight hybrid rows.

The number of samples which could be gathered, weighed, and dried in the oven within a given period was limited, so that moisture determinations begun on June 3, 1924, were not completed until June 14. Rains occurred during this period, and the moisture content of the samples gathered near its close was slightly higher than that of those gathered at the beginning. The results are shown in Table 19.

The Kanred wheat had an average moisture content of  $79.12 \pm 0.18$  per cent. The Minhardi averaged  $76.91 \pm 0.21$  per cent moisture. The moisture content of the hybrids extended over a range practically the same as that of the two parents combined. The number of rows of both parents and hybrids, however, was not sufficient to give particular significance to the range of variability. The hybrids show a fairly close approximation to a normal frequency distribution when grouped into classes differing in moisture content by 1 per cent. The variability of the parental rows, however, prevents an interpretation

of the mode of inheritance of moisture content in this experiment. The difficulty of determining the inheritance of moisture content, one of the qualities influencing hardiness, the results of which determination were shown in Table 19, tends to show that hardiness itself is not readily analyzed genetically.

TABLE 19.—*Frequency distribution of the moisture contents of plants of Kanred, Minhardi, and Kanred-Minhardi F<sub>3</sub> hybrid winter wheats at University Farm, St. Paul, Minn., in June, 1924*

Moisture content classes	Number of rows		
	Kanred	Minhardi	F <sub>3</sub> hybrid lines
73.0-73.9 per cent			2
74.0-74.9 per cent		2	0
75.0-75.9 per cent		1	10
76.0-76.9 per cent	1	7	32
77.0-77.9 per cent	2	6	33
78.0-78.9 per cent	6	2	35
79.0-79.9 per cent	4	1	30
80.0-80.9 per cent	5		9
81.0-81.9 per cent	1		1
82.0-82.9 per cent			
83.0-83.9 per cent			
84.0-84.9 per cent			
85.0-85.9 per cent			
Total	19	19	152
Mean moisture content	79.12±0.18	76.91±0.21	77.87±0.08

## DISCUSSION

The results here presented show many irregularities which, of course, make the drawing of accurate conclusions more difficult than with more uniform data. The studies, however, have brought out many points which must be taken into consideration in formulating the principles involved in hardiness in wheats. Using these studies, as well as the investigations of others, as a basis, the characteristics of hardiness and hardening in wheats and the factors influencing them may be considered.

### CHARACTERISTICS OF HARDY VARIETIES

When growing actively in the fall or spring, hardy varieties of wheat as compared with nonhardy varieties are characterized by low moisture content and juice having a high percentage of total solids and a high freezing-point depression. The freezing-point depression and total solids in the juice are closely correlated, and both vary inversely with the moisture content of the tissue. Any variety of winter wheat in an unhardened condition will survive a temperature of  $-5^{\circ}\text{C}$ . for 24 hours, but none will survive  $-10^{\circ}\text{C}$ . for the same period. A hardening process is necessary, therefore, to permit wheat to survive the low winter temperatures which occur in the Northern States. Wheat plants without snow protection have survived temperatures of  $-25^{\circ}\text{C}$ . or lower.

After hardening has occurred, the previously mentioned differences in moisture content and juice concentration between hardy and nonhardy varieties may not hold, or the relationship may be reversed. The hardy and nonhardy varieties then differ chiefly in imbibitional pressure of the cell colloids, as determined by the ability of wheat leaves to hold their moisture against the forces of

freezing and pressure. Hardy rye in an unhardened condition has a moisture content and juice concentration similar to those of a non-hardy wheat, but when hardened it has a greater imbibition pressure than the hardest wheat.

At low temperatures, hardy varieties of rye and wheat have a lower rate of respiration than nonhardy wheats and thus retain their sugars longer or to a greater extent.

#### CHANGES OCCURRING DURING HARDENING

When growth is retarded by low temperature the sap concentration increases, due to the formation of sugars from starch and, after freezing, to the splitting of proteins into amino acids, as shown by Newton.<sup>7</sup> The sugars and some salts partly protect the protoplasmic colloids from being coagulated by freezing, according to Newton<sup>7</sup> (35, 36) Maximow (27), Schaffnit (48), and Schander and Schaffnit (50). Freezing of the plants causes water to be withdrawn from the cells. Harvey (19) found that on thawing much of this water is evaporated, leaving the cell sap more concentrated than before freezing. Thus the moisture content of wheat leaves decreases during the autumn. With a decrease in moisture content and an increase in sugars and amino acids, the total solids increase and the freezing point of the juice is lowered. This lowering of the freezing point is of little consequence in itself, but the increase in solutes decreases the formation of ice and may protect the protoplasm from precipitation.

When the sap concentration increases, the resistance to freezing of the plant tissue increases at a still greater rate, as noted by Chandler (9). Apparently the increased juice concentration results in an increased imbibition pressure of the cell colloids, which decreases the withdrawal of water from the cells by freezing or pressure. Probably the increased imbibition pressure is the result of greater dispersion of the protoplasmic proteins. According to Petit (41), imbibed or capillary water permits more undercooling before ice is formed.

Hardy varieties having a greater initial juice concentration possess a slightly greater resistance to freezing injury, probably because less water is withdrawn from the cell in freezing. At low temperatures, hardy varieties form more sugar and increase the sap concentration more than nonhardy varieties. More of the plant tissues of hardy varieties also remain active after a freeze, even with low moisture content. The greater juice concentration in the living cells of the hardy plants causes a greater imbibition of cell juice by protoplasmic colloids. This, together with the greater protection against coagulation of the protoplasm by freezing, due to sugars, accounts for the cold resistance of certain varieties of wheat and rye. The more hardy the variety the more readily is it able to increase the imbibition pressure of the tissues.

The temperatures or combination of temperatures best suited for hardening in wheat are not known, and their determination should furnish a fertile subject for investigation. Results reported here show that gradual reductions in temperature increase hardiness and that exposure to 0° C. is more effective than exposure to temperatures

<sup>7</sup> NEWTON, R. Op cit.

of 5° C. or -5° C. Probably alternations of freezing and thawing are most effective.

The time required for maximum hardening also is not known. Rein (42) reported that maximum hardening occurred in some plants in 6 to 8 days. Winkler (57) reported that trees can be hardened by a 5-day exposure to low temperature even in the summer.

When the temperature rises above freezing, water can enter the plants and also the cells if the protoplasm has not been killed. This increase in moisture decreases the juice concentration. At a sufficiently high temperature the sugars will revert to starch and the amino acids will form proteins. The imbibition pressure will then decrease, and the tissues will be less hardy. Rather marked differences have been observed between successive collections made three or four days apart. A decided difference in rye was observed between the collections of November 17 and 18, 1925, and a still more abrupt difference was observed between the Kanred wheat collected in the morning and that collected in the afternoon of November 5. However, the later collections of both rye and wheat were in the laboratory three or four hours while the plant parts were being separated, which might account for some of the changes in these instances.

A freeze during the night, followed by thawing, will increase the quantity of juice that can be expressed from the leaves. After the evaporation of the melted ice crystals, less juice can be expressed. If a freeze is not too severe, the plant hardens upon thawing, but after a hard freeze the protoplasm is coagulated and dehydrated to such an extent that it can not take up water and the plant is killed instead of hardened.

With different freezing temperatures affecting hardy and nonhardy varieties somewhat differently, and with various degrees of recovery or response to temperatures above freezing, the irregularity in the winterkilling of wheats is not surprising. Adjoining rows of wheat show rather wide differences in the imbibition pressure of the tissues. Doubtless there are nearly as great differences between plants within a row. If so, this further explains the irregularities in killing which usually occur and which can not be explained by differences in protection of the plants.

#### THE PRACTICAL MEASUREMENT OF HARDINESS

The results of these studies indicate that no laboratory method yet devised, except perhaps the controlled freezing, is any more accurate for determining hardiness than is careful field study. The moisture content of unwilted wheat leaves probably is only one of the factors concerned in hardiness and is itself subject to considerable variation. When wilted tissue is present, moisture content is not a dependable character, as tender varieties are wilted more than hardy ones. Under ideal conditions, however, the moisture content is a valuable index of hardiness in wheat leaves.

The imbibition pressure in wheat leaves may show a striking relationship to hardiness during late autumn, but even then it is highly variable from day to day. When the tissue remains frozen during the winter, or when the plants freeze while in a growing condition, the imbibition pressure, as measured by the quantity of expressed juice, is of little value in determining hardiness. This test requires

considerable labor and equipment at all times; therefore, in view of its variability, it can not be expected to serve as a practical measure of hardiness in wheat except under ideal conditions such as are obtained in certain seasons.

Total solids, freezing-point depression, and bound water in the juice are related to hardiness only under certain conditions and are of little use for practical measurement of hardiness except early in the autumn, when the total solids and freezing-point depression are inversely proportional to hardiness. The bound-water content of the juice from macerated tissue should measure the imbibition of the protoplasmic colloids, but its determination is more tedious than that of ordinary freezing and pressing.

Freezing under controlled temperatures offers the greatest promise in measuring the hardiness of wheat plants by laboratory methods, even though the cold resistance of the plants does vary considerably. A rough measure of hardiness can be obtained without any previous hardening, but for more accurate measurements the plants must be subjected to successively lower temperatures. Refrigeration equipment, although expensive, probably would be the most economical means of accurately measuring cold resistance, if an exact technic in hardening were developed. The next most feasible method is by growing the varieties in replicated rows at selected field stations. The plants can be protected somewhat by sowing the seed between rows of grain stubble where conditions are rather severe, or protection can be decreased by thin sowing.

#### SUMMARY

The studies here presented are concerned only with low temperatures and not with killing due to winter drought, soil blowing, heaving, or smothering by ice.

Hardy wheats are characterized by a low moisture content of the tissues, a high percentage of total solids in the juice, a high freezing-point depression or osmotic concentration of the juice when the plants are actively growing, a high percentage of bound water in the juice, a low rate of respiration at low temperatures, and frequently by a long period of vegetative growth.

The most important character influencing hardiness is the ability to build up a high imbibition pressure of the cell colloids during hardening.

Swedish (Minnesota No. 2) rye, which is more hardy than any wheat, has a high moisture content, a low percentage of total solids, and a low freezing-point depression like nonhardy wheats, but it has a lower rate of respiration at low temperatures and a greater imbibition pressure than the hardest wheats.

During the hardening of wheats there is a decrease in moisture content and an increase in total solids in the sap, freezing-point depression of the sap, and imbibition pressure of the cell colloids, as measured by the ability of the tissues to hold sap against the forces of freezing and pressure.

In the tissues of a given variety, the quantity of juice expressed is positively correlated with the moisture content. The percentage of total solids and the freezing-point depression of the juice are negatively correlated with moisture content and the quantity of

juice expressed from the tissues. The quantity of juice retained after pressing is positively correlated with moisture content in unfrozen tissues, but not significantly so in frozen tissues.

The moisture content, sap concentration, imbibition pressure, and hardness of wheat and rye plants fluctuate widely during the fall and winter, and differences between varieties do not exist at all times.

The freezing of plants at controlled temperatures, followed by a determination of the extent of killing, appears to be the most feasible laboratory method for measuring hardness.

A sudden exposure of wheat and rye plants from the greenhouse to a temperature of  $-10^{\circ}$  C. for 24 hours will kill the hardiest varieties. Any variety of winter wheat will withstand a temperature of  $-5^{\circ}$  C. for the same period. Hardening is necessary to protect wheat against the much lower temperatures than  $-10^{\circ}$  C. which occur in northern wheat-growing areas. The harder the variety the more it can harden.

The crown is the most hardy portion of the wheat plant above the soil surface. Young leaves are more hardy than old leaves, and the bases of leaves are more hardy than the tips.

The genetic factors involved in the inheritance of hardness have not been determined, but most  $F_3$  strains are intermediate between the parents in hardness. Accurate measures of hardness in the field are difficult because of the variations both in the plants and in weather conditions.

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# THE SEEDS OF QUACK GRASS AND CERTAIN WHEAT GRASSES COMPARED<sup>1</sup>

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## INTRODUCTION

Because of the great damage to farm land caused by quack grass in northern and eastern United States, it is important that the seed analyst be able to identify positively quack grass seed<sup>2</sup> when it occurs in forage crop seed. Occurring as an impurity in seeds of the coarser grasses and especially in seed of awnless brome grass (*Bromus inermis* Leyss), quack grass seeds are easily confused with seeds of western wheat grass (*Agropyron smithii* Rydb.) and slender wheat grass (*Agropyron tenerum* Vasey). Descriptions of the seeds of the three species of *Agropyron* have already been published by Pammel and King, Sarvis, Bolley, and Dahlberg.<sup>3</sup> Hillman<sup>4</sup> has described the spikelets in detail as they appear in commercial lots of seed. Sarvis has contributed a key to the seeds based on the pubescence and glands on the palea and rachilla segment. Dahlberg, in the most comprehensive paper heretofore published on the subject, has described briefly the differences in general form, in the shape of the rachilla segment and the outline of the palea tip, and has compared the number and kind of hairs on the rachilla segment, palea, and lemma.

Nothing need be added to what has already been written concerning the pubescence on the seeds of these three species, but for the benefit of those not equipped with magnifiers of sufficient power (35 diameters or more) more consideration may be given to the differences in general form of the seed and to the differences in form exhibited by the different parts of the seed. Frequently an examination of the structural characters is sufficient to determine the species.

The writers describing individual seeds of the three species state clearly that there is no one character that can be relied upon as a means of identification. This is necessarily true because the characters which have to do with quantity and kind of hairs vary greatly, even between individual seeds of the same sample, and the characters, such as shape of rachilla segment and outline or shape of seed, depend somewhat on the maturity and the position of the seed in the spikelet. A determination of species requires familiarity on the part of the analyst with the variations both in pubescence and in general form.

<sup>1</sup> Received for publication Apr. 28, 1927; issued November, 1927.

<sup>2</sup> The word "seed" in this paper is used in the popular sense and refers to the ripened floret.

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It is the purpose of this paper to point out graphically the differences in form and appearance as exhibited by the seed as a whole and by its component parts. For the convenience of the analyst, an illustrated tabulation of the differences in pubescence as described by Dahlberg, together with explanatory text, is also given.

In the absence of a single constant diagnostic character, those characters which are displayed in the greatest number of seeds of a species are the ones a study of which will prove most helpful. Familiarity with all of the characters of a species and the corresponding characters in the other species will enable one to identify the average seed.

In addition to the characters generally true of a species, there are others to be found in occasional seeds, the counterpart of which can not be found in the seed of the other two species. A knowledge of these extremes in form peculiar to one of the three species is also helpful.

### CHARACTERS GENERALLY TRUE OF A SPECIES

#### GENERAL FORM

Seeds of *Agropyron repens* are narrowly boat shaped in outline and acutely pointed at the apex. They are thick, somewhat flattened dorsoventrally, with a ventral groove the entire length of the grain. The palea in conforming to the shape of the grain is broadly concave throughout its entire length.

Seeds of *Agropyron smithii* resemble those of *A. repens* in outline, but are more robust and are not flattened dorsoventrally at the callus to the degree evident in seeds of *A. repens*. The palea is more deeply concave.

Of the three species here compared, the seeds of *Agropyron tenerum* are the most easily recognized by their general form. They differ from seeds of *A. repens* and *A. smithii* in having their greatest width at a point a little above the middle and in having a much thinner grain, the ventral groove becoming very shallow toward the apex. This causes the palea to flatten out at the tip.

The bilateral displacement of the lemma and the lack of symmetry observed in many seeds of *Agropyron tenerum* also serve to differentiate a large proportion of the seeds of this species from those of the other two. The lack of symmetry is due to the unequal bending of the different parts of the floret to conform to the closely appressed position of the spikelet on the stem. The displacement of the lemma is a result of the curving of the upper portion of the lemma toward the stem of the spike, while the palea, being shorter and adherent to the rigid grain, remains straight. (Fig. 1.)

#### COLOR AND TEXTURE

Seeds of *Agropyron repens* found in brome-grass samples are generally light yellow tinged with green, while the seeds taken from well-matured spikes have a brownish tinge, and sometimes are a darker shade above the callus on the dorsal side. The palea and the lemma are thick and lustrous.

Mature seeds of *Agropyron smithii* often are deeper yellow and the lemma and palea sufficiently thin to show the dark-colored grain. In fresh unrubbed seed, the surface of the lemma and palea is not lustrous as in the other two species.

The lemma and palea of *Agropyron tenerum* are pale flesh-colored, thin, and polished, and show very little of the greenish color so prevalent in seeds of *A. repens*.

#### TIP OF PALEA

The outline of the tip of the palea is worthy of consideration even though in *Agropyron repens* and *A. tenerum* there is considerable variation.

In *Agropyron repens* the tip of the palea is usually truncate or indented, occasionally obtuse or rounded. The broadly truncate type has not been observed by the writer in either of the other two species.

The tip of the palea of *Agropyron smithii* is either indented or cleft. The deeply cleft tip which is frequently found in this species is rarely found in the other two species.

In *Agropyron tenerum* the tip of the palea is either narrowly truncate, rounded, or indented. (Fig. 2.)

#### RACHILLA SEGMENT

While the length and breadth of the rachilla segment vary within the species according to the position of the seed in the spikelet, the divergence of the sides of the rachilla segment keeps within certain limits for each species and when considered with other characters serves to identify the seed.

The sides of the rachilla segment of *Agropyron repens* diverge slightly in the lower seeds of the spikelet, but in those taken from the middle and upper part of the spikelet the sides of the rachilla segment are approximately parallel. In all seeds the sides of the rachilla segment are straight, and the segment appears flattened against the palea, either within the intumed keels or against them.

The sides of the rachilla segment of *Agropyron smithii* diverge noticeably except in some terminal seeds, and the lines of divergence, instead of being straight as in the other two species often curve out at the upper end. The segment is not appressed to the palea.

The sides of the rachilla segment of *Agropyron tenerum* diverge slightly in all except terminal seeds. The rachilla segment is not appressed to the palea, and in many seeds the segment is inclined laterally following the slight curvature of the seed. The long hairs on the rachilla segment of *Agropyron tenerum*, visible with a hand lens, serve to distinguish a large proportion of the seeds of this species. (Figs. 2 and 3.)

#### LEMMA

It will be noted that in well-developed seeds of *Agropyron repens*, with the exception of those developed from basal florets, the marginal areas of the paleas are not wholly covered by the lemma. In the seeds of the other two species, the lemma generally extends to the keels of the palea. (Fig. 1 and 2.)

Examination of the sinus, or the opening between the edges of the lemma at the base of the rachilla, is helpful in distinguishing the seeds of *Agropyron repens* from those of the other two species.

In *Agropyron repens* the sinus is essentially U-shaped, the edges of the lemma not obscuring or overlapping the sides of the rachilla segment at its base.

The sinus in *Agropyron smithii* and *Agropyron tenerum* is more V-shaped and the edges of the lemma at the callus obscure the sides of the rachilla segment.

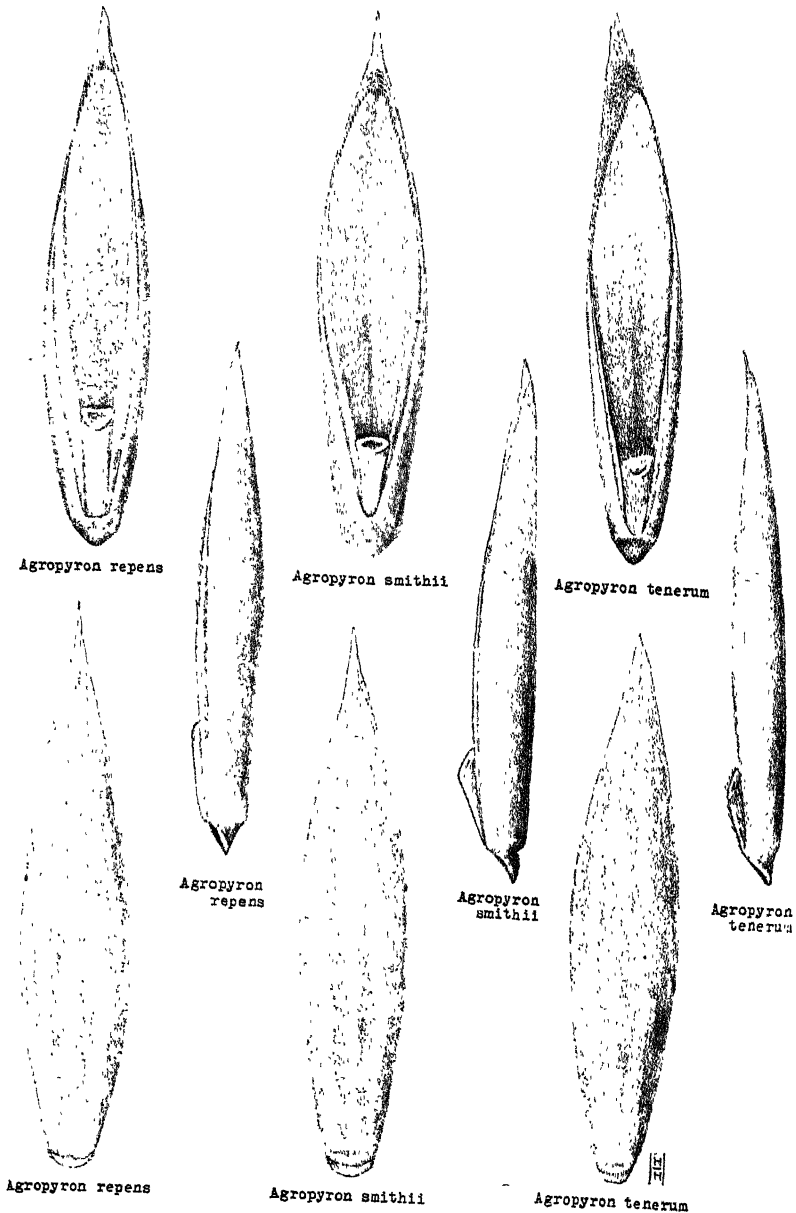


FIG 1.—Dorsal, lateral, and ventral view of the seeds of three species of *Agropyron*.  $\times 7\frac{1}{2}$

In some basal seeds of *Agropyron smithii* the sinus is so narrow and the rachilla segment so short that only the top of the segment is visible. (Fig. 3.)

## FREE GRAINS

The free grains of the three species resemble one another closely, and there is no way known to the writer by which the species can be determined. Grains of the three species are generally light brown,

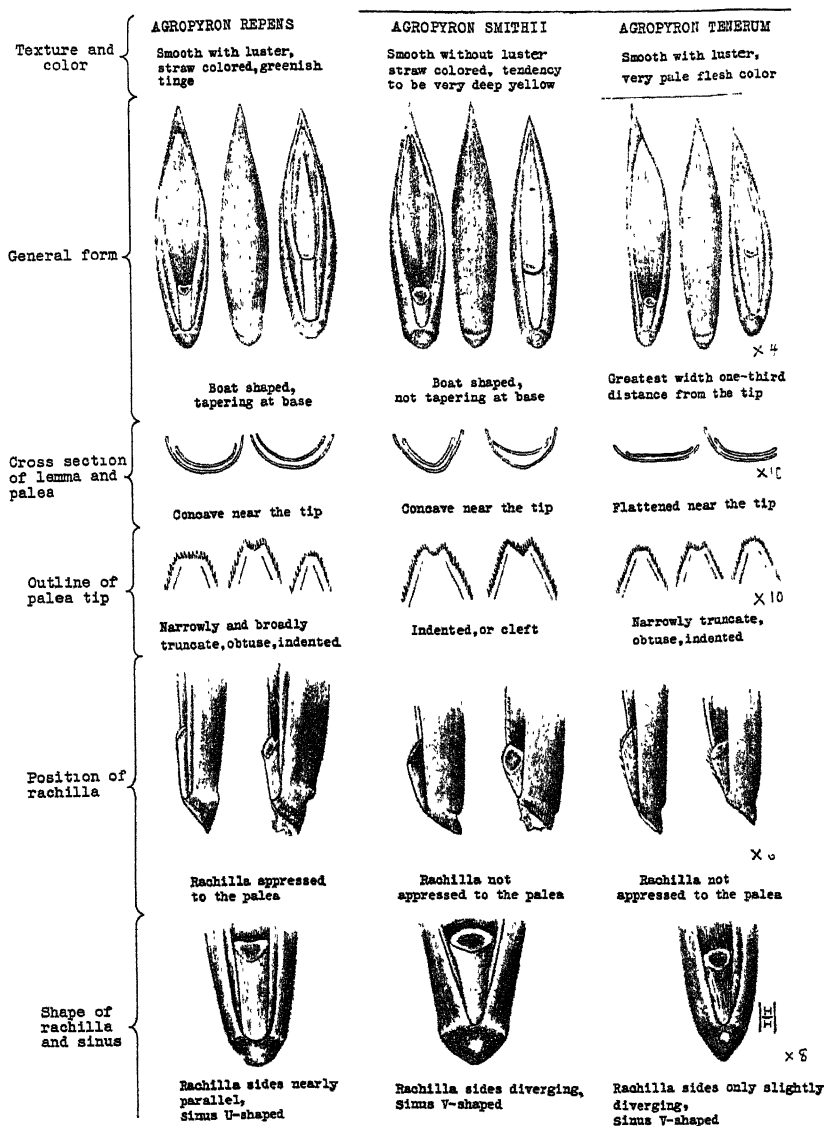
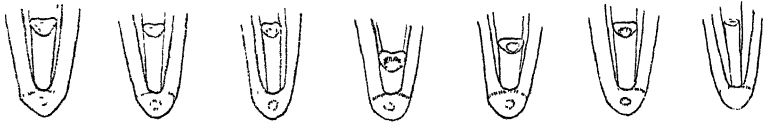


FIG. 2.—Diagnostic characters of the seeds of three species of *Agropyron*, showing differences in general form and appearance

those of *Agropyron smithii* being sometimes very deep purple. The grains of *Agropyron tenerum* are thinner and longer than those of the other two species, and the ventral groove becomes only slightly concave at the upper end.

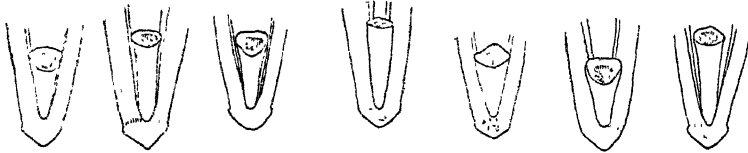
For the convenience of the analyst, the diagnostic characters which are generally true of each species are listed in an illustrated tabulation (fig. 2).



*Agropyron repens*, seed from middle and upper part of spikelet



*Agropyron repens*, basal seeds



*Agropyron smithii*, seed from middle and upper part of spikelet



*Agropyron smithii*, basal seeds



*Agropyron tenerum*, seed from middle and upper part of spikelet



*Agropyron tenerum*, basal seeds

FIG. 3.—Basal portions of the seeds of three species of *Agropyron*, showing the position of the lemma in relation to the rachilla

#### CHARACTERS AS MODIFIED BY IMMATURITY AND POSITION OF SEED IN SPIKELET

The ease with which the individual seeds of the three species can be determined depends somewhat on the maturity of the seeds and whether or not the seeds represent terminal florets.

Terminal seeds are difficult to determine as to species because of the straplike elongation of the rachilla and the shortening of the lemma. When immature, the seeds of the three species resemble one another so closely that an examination of the pubescence is necessary for a safe determination.

Seeds developed from basal florets when found free or inclosed in the glumes are easily identified. Although the rachilla is shorter and the lemma longer than in seeds from the upper part of the spikelet, the diagnostic characters are clearly evident. A knowledge of the differences in the glumes described by F. H. Hillman<sup>5</sup> enables one to identify easily seeds inclosed in the glumes. Because of the constant adherence to the stem of the spikelet, the lowest floret of *Agropyron repens* and *A. smithii*, when found free, generally shows evidence of being torn at the callus.

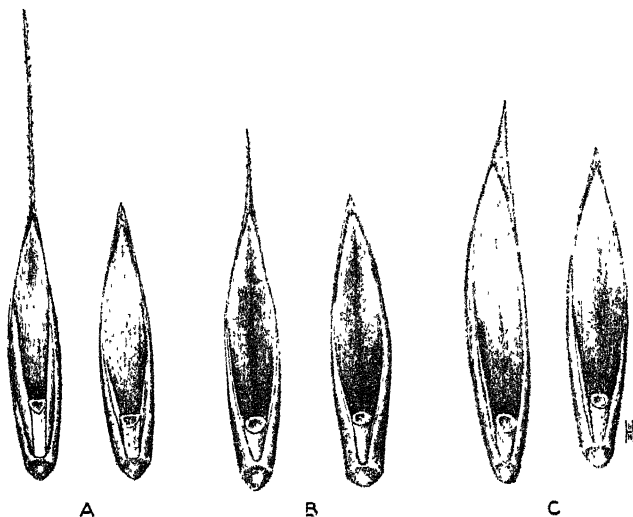


FIG. 4.—Seeds of three species of *Agropyron*, showing length of awn and acutely pointed lemmas: A, *Agropyron repens*; B, *A. smithii*; C, *A. tenerum*.  $\times 4$

#### CHARACTERS POSSESSED BY OCCASIONAL SEEDS OF A SPECIES

The characters exhibited by occasional seeds of *Agropyron repens* which are not duplicated in seeds of *A. smithii* and *A. tenerum* are the long awn, the longitudinal wrinkle in the palea, and the pronounced bulge at the base of the lemma.

Seeds of the long-awned form of *Agropyron repens* are found frequently in awnless brome-grass seed. Figure 4 shows the long-awned form of *A. repens* and the acuminate tipped lemmas of *A. smithii* and *A. tenerum*. Botanists have reported a long-awned form of *A. tenerum* growing in high altitudes in the Colorado Rockies, but it is very improbable that seeds of this plant would ever occur in trade samples. Seeds resembling those of *A. tenerum*, having a very long awn, frequently have been found in awnless brome-grass seed from Canada. These have been identified as *A. caninum* (L.) Beauv., a closely allied species. Aside from the long awn, seeds of

<sup>5</sup> HILLMAN, F. H. Op. cit.

*A. caninum* differ from those of *A. tenerum* in having longer hairs, both on the rachilla and around the callus.

The longitudinal fold or wrinkle in the palea is shown in Figure 5. Seeds of *Agropyron repens* having this wrinkled palea are not of frequent occurrence in forage-crop seed, but a sample of *A. repens* recently examined contained a considerable number of seeds of this type. All having this type of palea were well-developed seeds.

The pronounced bulge at the base of the lemma just above the callus, observed in some well-developed seeds of *Agropyron repens*, is shown in side profile in Figure 6, with the corresponding view of the seeds of the other two species. Not only is the bulge less conspicuous in *A. smithii* and *A. tenerum*, but the back edge of the callus is very nearly in line with the back of the lemma, while in the seeds of *A. repens*, figured, the back edge of the callus is coincident with the vertical axis of the seed. In the seeds of *A. repens*, here figured, and occasionally in seeds of *A. tenerum*, the lemma is not indented below the bulge. This is in marked contrast to the lemma of *A. smithii*, which apparently always is abruptly indented or broken at this point. (Fig. 6.)

A character observed in seeds of *Agropyron smithii* and apparently not appearing in seeds of *A. repens* or *A. tenerum* is the deep groove in the deeply concave palea.

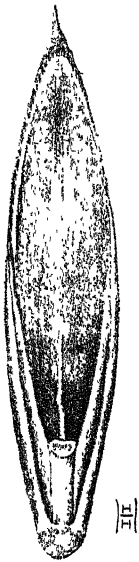


FIG. 5.—Seed of *Agropyron repens*, showing wrinkled palea.  $\times 7\frac{1}{2}$

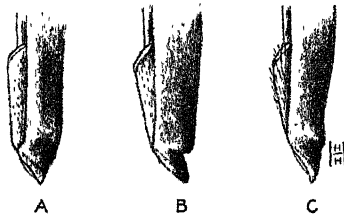


FIG. 6.—Side view of basal portion of seeds. A, *Agropyron repens*; B, *A. smithii*; C, *A. tenerum*.  $\times 7$



FIG. 7.—Seed of *Agropyron smithii*, showing deep groove in palea.  $\times 7$

Figure 7 shows a seed of this type taken from the upper part of the spikelet. These should not be confused with seeds of *A. tenerum*, which have a shallow groove in a broadly concave palea.

#### DIFFERENCES IN PUBESCENCE AS DESCRIBED BY DAHLBERG

Figure 8 shows the differences in pubescence, as described by Dahlberg.<sup>6</sup> The following is from his paper, his figures and references to them being omitted:

##### RACHILLA

The hairs clothing the rachilla constitute a valuable character used in the determination of the seed. However, care and good judgment must be exercised because of the great variation which may occur.

The characteristic rachilla of *A. repens* is sparsely covered with short, minute hairs having a rather large base. Occasionally a glandular structure may be

<sup>6</sup> DAHLBERG, R. C. IDENTIFICATION OF THE SEEDS OF SPECIES OF AGROPYRON. Jour. Agr. Research 3; 275-282, illus. 1914.

discerned at the base. This, however, can only be seen with a high-power lens and is not considered of sufficient importance to warrant its use as a determining character. No rachilla of *A. repens* has been found which had the hirsute character of *A. smithii* or the pilose character of *A. tenerum*.

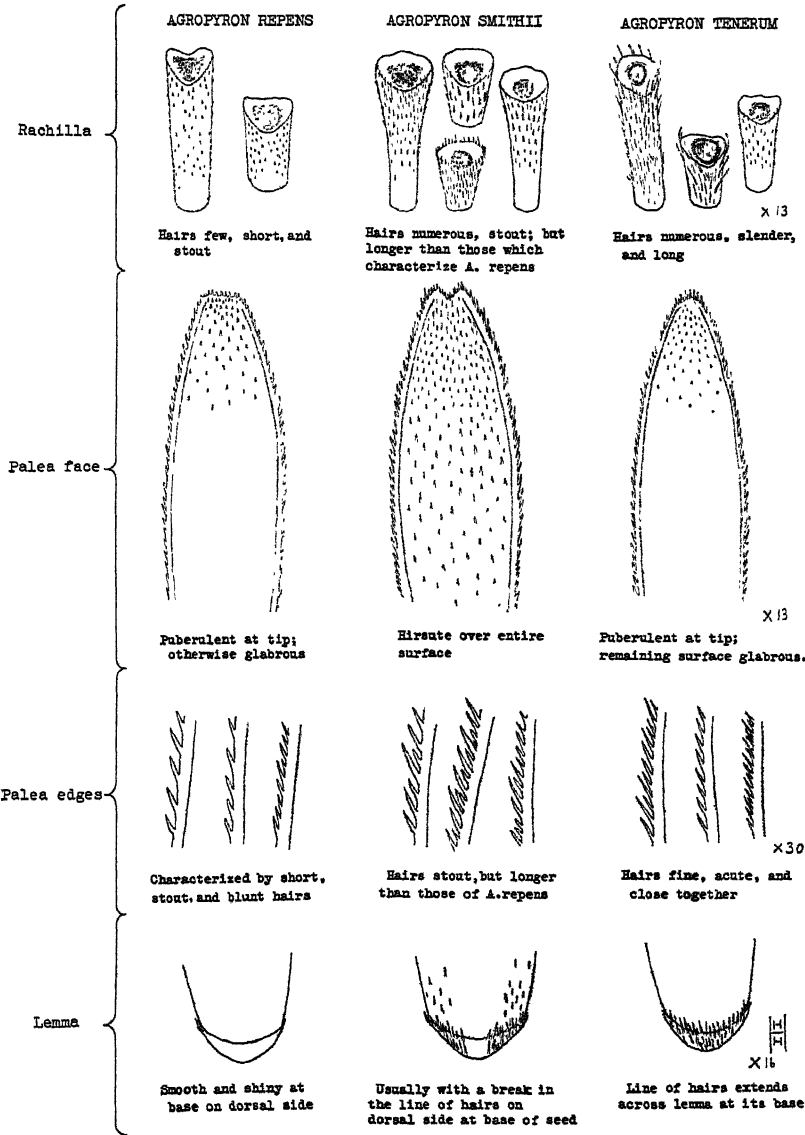


FIG. 8.—Diagnostic characters of seeds of three species of *Agropyron*, showing differences in pubescence, as described by Dahlberg

The rachilla of *A. smithii* is characterized by hairs of the same general shape as the hairs found on the rachilla of *A. repens*. They are, however, larger and stronger and the number is noticeably greater. This characteristic is fairly uniform.

The rachilla of *A. tenerum* is characterized by hairs of a pilose nature. They are long as compared with those of *A. repens* and *A. smithii*, and may often be

distinguished by this feature alone from these two species, as the pilose nature has never been observed on them. However, an absolutely authentic specimen of *A. tenerum* has been examined which had a rachilla much resembling that of *A. repens*. The hairs were short, but were not as large as the base. Other characters on the seed, however, made it possible to place it accurately in the species *tenerum*.

#### LEMMA

Another distinguishing character and one which is reliable as to uniformity may be found at the base of the lemma on the ventral<sup>7</sup> side of the seed. In *A. tenerum* there is a line of hairs which extends from the base of the rachilla on the dorsal side of the seed around and entirely across the face of the lemma on the ventral side near the base of the seed. In some cases it may be impossible to distinguish the hairs on the middle of the lemma, but the surface of the lemma at this point is roughened sufficiently so that it is noticeable. This is a fairly definite character.

The seed of *A. repens* has no such characteristic line of hairs, but the basal portion of the lemma is entirely smooth and shiny. This character in the seed of *A. smithii* is somewhat variable and is therefore not of much value. Most commonly, however, it is found that the ring of hairs extends part way around on either side, and on the middle of the lemma there is a space which usually is entirely smooth.

#### PALEA

The part of the seed which discloses good and reasonably definite characteristic differences is the palea. The face of the palea in *A. repens* and *A. tenerum* is practically glabrous, except near the tip, where it is puberulent. Occasionally there is a small number of hairs distributed over the face of the palea. Since the tips of the palae in both of these species are always puberulent, this can not be used as a distinguishing character. The palea of the seed of *A. smithii* is quite hirsute over its entire surface.

The hairs on the edge of the palea have a distinctive shape for each of the three species and are very useful as a determining factor. Those of *A. repens* are rather short, stout, and somewhat blunt. Those of *A. smithii* are about as coarse as those on *A. repens* but are noticeably longer, thus making them appear more slender. On *A. tenerum* the hairs are finer, closer together, and more acutely pointed than in the case of the two others.

#### SUMMARY

The individual seeds of *Agropyron repens*, *A. smithii*, and *A. tenerum*, with the exception of some terminal or immature seeds, can be identified as to species by a study of the characters, such as form, color, texture, concavity of palea, outline of palea tip, the shape of the rachilla segment, and the width of the opening between the edges of the lemma at the base of the rachilla.

In identifying seeds of quack grass (*Agropyron repens*) the shape of the rachilla and the width of the opening between the edges of the lemma at the base of the rachilla segment are valuable diagnostic characters.

Familiarity with the extremes in form observed in occasional seeds of a species but not duplicated in either of the other two species enables the analyst to identify seeds of these types at sight.

With the combined study of the differences of general form and the differences in quality and quantity of pubescence, all individual seeds of *Agropyron repens*, *A. smithii*, and *A. tenerum* should be easily identified.

<sup>7</sup> The writer evidently means dorsal instead of ventral. Terms interchanged also in following sentence.

# THE TOXIC CONSTITUENT OF RICHWEED OR WHITE SNAKEROOT (*EUPATORIUM URTICAEFOLIUM*)<sup>1</sup>

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## HISTORICAL REVIEW

Among the many perils and hardships that marked the settlement of the old frontier of the United States was the mysterious malady known as "milk sickness." Appearing customarily late in the summer it rapidly assumed epidemic proportions, sparing neither age nor sex, frequently sweeping away a quarter of the population, and sometimes depopulating whole villages. Nearly every district in the old West—now the Central West—suffered at one time or another from this disease and so sudden was its onset, so recondite its cause, so unavailing were the preventive measures taken by the terrified backwoodsmen, and so fatal were the attacks, that the old chroniclers refer to it in the strongest terms as formidable, terrible, and frightful. Nicolay and Hay (17)<sup>2</sup> describe a characteristic outbreak during which the mother of Abraham Lincoln died.

In the autumn of 1818 the little community of Pigeon Creek was almost exterminated by a frightful pestilence called the milk sickness, or, in the dialect of the country, "the milk sick." It is a mysterious disease . . . It seems to have been a malignant form of fever—attributed variously to malaria and to the eating of poisonous herbs by the cattle—attacking cattle as well as human beings, attended with violent retching and a burning sensation in the stomach, often terminating fatally on the third day. In many cases those who apparently recovered lingered for years with health seriously impaired.

The disease was known in Maryland, North Carolina, Kentucky, Tennessee, Alabama, Missouri, Illinois, Indiana, and Ohio from the time of the early settlements. Wolf, Curtis, and Kaupp (22) in one of the most complete accounts of this sickness that has ever been published, have shown that milk sickness was recognized as a particular disease in North Carolina before the Revolution. The early settlers were also acquainted with a disease of cattle known as "trembles" that appeared only in "milk-sick areas" and during the same periods in which human milk sickness was prevalent. It was observed, too, that suckling calves frequently sickened and died from "trembles" in these same milk-sick areas and that small domestic animals, pigs, cats, and dogs, fed on the milk of cows that were permitted to range the woods, developed symptoms of nausea, weakness, and enteritis, and died. These facts led to the suspicion that the milk of cows at certain seasons contained some deleterious substance that was capable of causing milk sickness in human beings who drank it or ate butter and cheese made from it. This idea was further strengthened by the fact that those who prudently abstained from all bovine products late in summer and fall were free from attacks of

<sup>1</sup> Received for publication May 12, 1927; issued November, 1927.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 575.

milk sickness and that those who discontinued eating milk products as soon as they began to sicken with the disease generally recovered after suffering merely a slight illness.

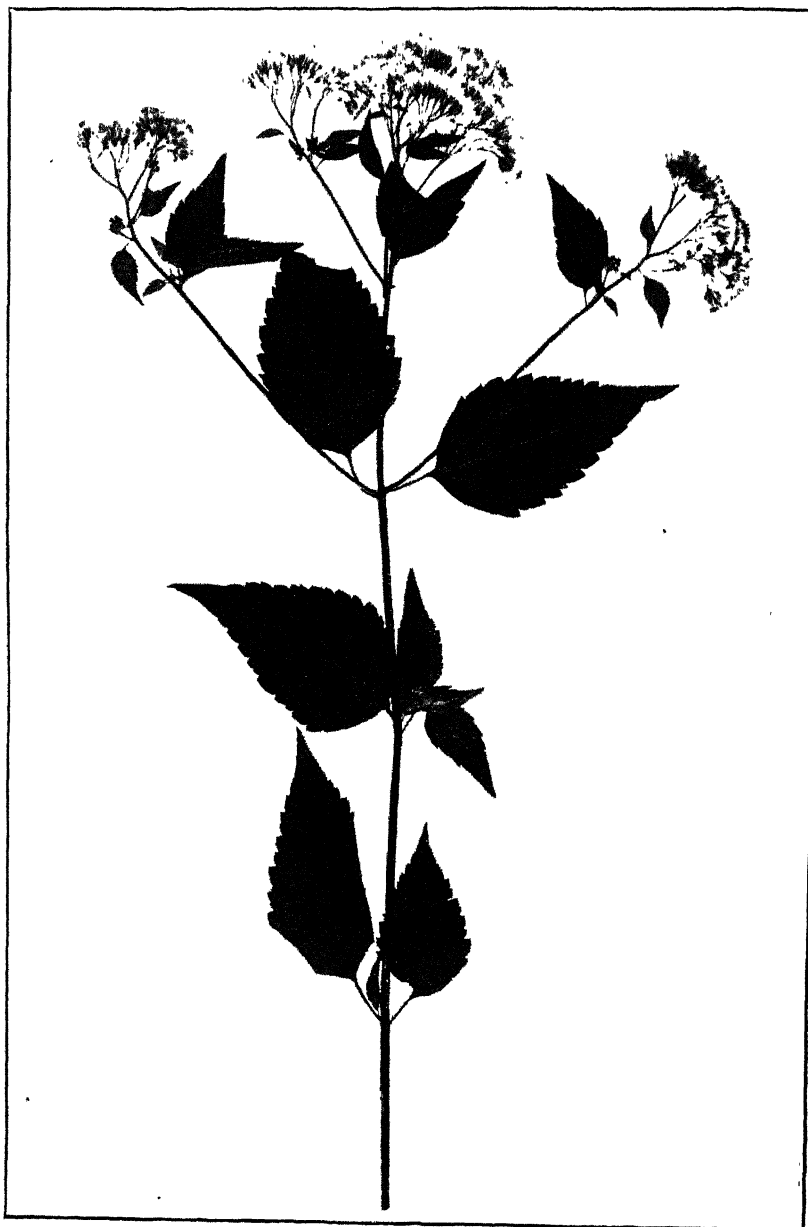


FIG. 1.—Richweed or white snakeroot (*Eupatorium urticaefolium*)

The nature of the deleterious substance present in this poisonous milk remained obscure. Speculation as to its character was widespread and resulted in the advancement of many fantastic ideas.

Poisonous dew and volatile minerals that evaporated from the earth at night, condensing on the herbage, there to await ingestion by the grazing herds, miasmata, the early conception of a germ theory, microorganisms, various poisonous minerals, arsenic, copper, lead, cobalt, as well as poisonous springs, all were advanced as explanations of the origin of the scourge. By far the most favored theory, however, and one held tenaciously from the earliest times was that milk sickness was caused by the feeding of the cows on some weed or herb that could transmit poisons to the milk. This hypothesis was strengthened by a large number of observed facts. The disease was most virulent late in summer and early fall, particularly after a period of drought, when wholesome forage plants had been consumed and the cattle were compelled to graze on plants that they refused when other feed was plentiful. Again, cattle that were pastured in inclosed fields where weed infestation was slight did not contract the trembles nor did their milk cause milk sickness. It was observed that the incidence of milk sickness diminished as the forests were cleared and the land brought under cultivation. Many different plants were suspected to be the cause. Some of them, such as poison ivy, water hemlock (*Cicuta maculata*), "Indian hachy," Indian tobacco (*Lobelia inflata*), Indian hemp (*Apocynum cannabinum*), Virginia creeper (*Parthenocissus quinquefolia*), cross vine (*Bignonia capriolata*), Indian currant (*Symphoricarpos orbiculatus*), marsh marigold (*Caltha palustris*), spurge (*Euphorbia esula*), fool's parsley (*Arethusa cynapium*), mushrooms, and richweed (*Eupatorium urticaefolium*) were well known as poisonous plants. Others argued in favor of the modified hypothesis that the disease was caused by parasitic fungi or by molds that grew on various plants.

The last-named plant, *Eupatorium urticaefolium*, known commonly as richweed, white snakeroot, pool wort, pool root, white sanicle, Indian sanicle, deer wort, squaw weed, white top, and steria, has been most widely and commonly held responsible for the disease. A great many experiments have been made in which this plant has been fed to animals with the result that trembles has been produced in a large number of cases. In recent years a mass of proof has been obtained to show that the plant is poisonous to livestock and that suckling animals may be poisoned by the milk secreted by animals that have eaten it.

Notwithstanding the many evidences of the poisonous nature of richweed and the many indications of its connection with human milk sickness, some observers have doubted its relation either to trembles or to milk sickness, and have advanced very strong arguments against the conclusion. They have pointed out that cattle have been known to graze for long periods in places where richweed grows abundantly without the appearance of any cases of trembles in the cattle or of milk sickness in the people who drank their milk. They further contended that in many of the feeding experiments the animals failed to develop abnormal symptoms. Finally, it was discovered that a disease resembling trembles in cattle and milk sickness in human beings exists in certain places in New Mexico and Texas where richweed is not found.

It is quite true that cattle have been known to graze with impunity in pastures infested with richweed, but it is also true that stock do not voluntarily eat this plant so long as other forage is available;

and the mere presence of richweed in a pasture does not prove that it has been eaten by the animals confined there.

Failure to produce the disease by feeding the plant is inconclusive evidence unless supported by a thorough and systematic course of experiments. One feeding of *Eupatorium urticaefolium*, unless in very large quantity, would certainly fail to provoke symptoms of trembles, and the result of such a feeding may lead to an uncritical opinion that the plant is harmless. Feeding of dried or partly dried plant would also fail to produce the disease in many if not in all cases. Furthermore, it is by no means certain that there is not a variation in the toxicity of richweed at different seasons or in different years or in plants from different localities.

The discovery of endemic milk sickness in the Pecos Valley of New Mexico, where *Eupatorium urticaefolium* does not grow, was considered strong evidence against the conclusion that richweed caused that disease. It has been shown, however, by a number of investigators that milk sickness and trembles in New Mexico are caused by the rayless goldenrod, *Aplopappus heterophyllus* (A. Gray) (Blake), also termed *Isocoma wrightii*, *Isocoma heterophylla*, *Bigelovia rusbyi*, and *Bigelovia wrightii*.

The theory that milk sickness is due to some specific microorganism has been held by several observers (11). Jordan and Harris (9), in a very careful and detailed examination of the possibility of pathogenic microorganisms being the cause of the disease, isolated a bacillus that appeared to be a possible solution to the problem. This organism they named *Bacillus lactimorbi* and they made an extensive study of it. At the end of their research, however, they candidly stated (9, p. 484-485):

"Taken as a whole the facts do not surely indicate that a specific microorganism is the cause of milk sickness or trembles."

The work of Jordan and Harris was supplemented by a bacteriological study of their organism by Luckhardt (10) in which various strains of the organism fed by him to dogs did not produce typical trembles. In pathogenicity the organism was very erratic. Luckhardt found it very widespread and growing on plants, such as alfalfa, that have never been associated with milk sickness. His study was, as he says, "far from being decisive in establishing *B. lactimorbi* as the etiological factor in the production of milk-sickness."

At the present time the only hypothesis that has any standing is that richweed poisoning causes this disease in the East. All the experimental work reported in the last decade supports this point of view and demonstrates that *Eupatorium urticaefolium* is a distinctly poisonous plant and that milk obtained from cows that have fed on it is capable of producing the disease in other animals.

Full descriptions of the history and characteristics of the disease have been published (11, 8, 2, 18, 22).

Apparently the first published account of milk sickness was included by Daniel Drake (4), who published the information concerning the disease that Barbee, of Virginia, collected while visiting the Mad River country of Ohio, in 1809, and other data gathered by Drake himself. In a larger work (5) Drake again notices the milk sickness and mentions the fact that some ascribe it to the poison of some unknown plant that is secreted in cow's milk. Meanwhile Hall

(6) had published the case of Alex. Telford and family in which the milk from four cows was strongly suspected of causing the outbreak.

A great deal has been written about milk sickness in the medical journals and considerable controversial material has developed in which even the existence of the disease as a specific malady has been denied. Some cursory experiments were made to determine the toxic properties of richweed but they were often of such a nature as to yield little positive knowledge.

About 20 years ago Moseley (13) reported a series of experiments with laboratory animals that indicated toxic properties in richweed. Crawford (2) reviewed the work, made some experiments, and concluded that there is no relationship between richweed and milk sickness. The distinctly poisonous nature of the plant, however, was demonstrated beyond question by a series of researches published during the last 10 years by Curtis and Wolf (3), who showed that the disease was not infectious; by Marsh and Clawson (12); Wolf, Curtis, and Kaupp (22), who poisoned animals on the sap of the plant; and by Sackett (19), who was able to poison laboratory animals with extracts of the plants. The results of Sackett's experiments further confirmed the toxic character of richweed.

When the writer began the study of this subject in 1918, the principal problem to be solved was the isolation of the toxic constituent and the elucidation of its chemical properties.

#### PREVIOUS CHEMICAL INVESTIGATIONS OF RICHWEED

No complete chemical analysis has yet been made of the whole plant of *Eupatorium urticaefolium*, although a number of investigators in the course of studies of the toxic constituent, have made extracts of the plant and have fed these to animals.

Moseley (13) fed extracts of the plant in water and milk to rabbits and dogs, and also gave parts of the plant to these animals. Crawford (2) injected aqueous extracts subcutaneously into two rabbits which died and into three others which survived. These extracts were made from dried plants. Extracts from plants preserved in chloroform water failed to injure a cat, a dog, a sheep, and Crawford himself, when fed in small dosage. Injection of the ash, obtained from 10 and 20 gm. of dried plant and neutralized with acetic acid, into rabbits, did not produce poisoning.

In 1909 Moseley (14) published his conclusions that the toxic constituent of richweed is aluminum phosphate, and adduced experimental evidence in support of this hypothesis. In 1910 Moseley (15) published further evidence to support the aluminum phosphate hypothesis, and advocated the use of soda as an antidote.

Curtis and Wolf (3) fed aluminum phosphate in doses of from 2 to 16 gm. to two ewes during a period of 69 days, at the expiration of which each had received 412 gm. of the compound. Neither of the ewes developed any abnormal symptoms and each gained weight.

Mosely (16) withdrew the aluminum phosphate hypothesis in favor of the conclusion that an "ether soluble resin" is the toxic principle. He reported that he obtained 9 per cent of the dry weight of the leaves by extracting with ether and that nearly all the extract

was resin. He does not record his experiments but states that the resin poisons cats, rabbits, and guinea pigs like *Eupatorium*.

Wolf, Curtis, and Kaupp (22) in an excellent monograph describe the most extensive use of extracts that had appeared up to that time. Aqueous and alcoholic extracts fed by them to guinea pigs did not produce poisoning. One of three guinea pigs to which they fed hydrochloric acid extract died. The expressed juice of fresh plant was fed to five guinea pigs, four of which died. Dried extract from the sap prepared by exsiccation on a water bath at 100° C. did not affect three guinea pigs to which it was fed. The volatile matters from the sap fed to three guinea pigs did not affect the animals. The inspissated juice evaporated at a temperature less than 60° C. fed to three guinea pigs caused the death of one. The other two merely showed some depression. Five sheep were fed the expressed juice of richweed and died with symptoms of trembles; two sheep were fed inspissated juice evaporated at a temperature below 60° C. and died with symptoms of trembles; and a sheep was fed the nonvolatile portions of the juice and developed trembles and died. These investigators were able to produce trembles in two out of six suckling lambs by feeding richweed to the mothers. A sample of "milk-sick" butter was fed to three mice, two of which died. Seven dogs were fed meat from trembles cases and showed no ill effects. As a result of these experiments the investigators concluded that the toxic constituent of richweed is glucosidal. In all their experiments they used green plant collected at Shooting Creek, N. C.

A valuable contribution to the subject was made by Sackett (19), who conducted many experiments with extracts from the leaves of *Eupatorium urticaefolium* on rabbits and guinea pigs. Fresh and carefully dried leaves and alcohol and chloroform ether-ammonia (Prolius's solution) extracts of richweed fed by him produced poisoning in animals. The alcohol and Prolius's solution marcs were nontoxic as were the extracts made with salt solution and that from the roots. Viscera and meat from a rabbit killed by richweed poisoning did not affect a cat. An anaphylactic experiment was negative. Guinea pigs were not affected by the dried leaves.

Jordan, Whelan, and Gidley (?) made some experiments with extracts from dried plants collected in Indiana. These were fed to rabbits and the marc from a 0.1 per cent hydrochloric acid extraction was fed to a hen. The 50, 70, and 95 per cent alcohol extracts, an ether-chloroform extract, and the extract made with a menstruum of 50 per cent alcohol and 0.5 hydrochloric acid were nontoxic. A 0.5 per cent hydrochloric acid extract killed a rabbit in four hours. A 0.1 per cent hydrochloric acid extract was fed to a rabbit which died in three days; the marc from this fed to a hen produced no effect. A similar 0.1 per cent hydrochloric acid extract which had fermented produced no result when fed to a rabbit. The resin was nontoxic.

These investigators report that there is neither alkaloid nor glucoside in the plant.

Bukey (1) reported a partial analysis of the fruit of *Eupatorium urticaefolium*, which includes data concerning a drying oil that he extracted, together with a proximate analysis of the other constituents.

From this contradictory mass of data it may be concluded that: The toxic principle appears to be soluble in alcohol, ether, and chloroform, and insoluble in water; it is apparently nonvolatile and is destroyed or greatly weakened by drying the plant; it appears to be thermostable within reasonable limits; nothing is certain about its chemical characters.

#### THE PRESENT INVESTIGATION

The result of the present investigation has been the isolation of a definite, toxic substance from green *Eupatorium urticaefolium* which appears capable of producing the characteristic symptoms of trembles in sheep and of a second toxic substance, not yet obtained in a pure condition, which is toxic to guinea pigs but does not affect sheep even in very large doses and probably has no relationship to milk sickness.

#### TREMETOL

The substance which appears to be responsible for trembles is a viscous, oily liquid of pleasant, aromatic odor which does not solidify at very low temperatures and can not be made to crystallize. Analysis and molecular-weight determinations indicate that the formula of this substance is  $C_{16}H_{22}O_3$ . It has been named tremetol from tremere, to tremble, inasmuch as all feasible names derived from the generic name of the plant have already been used to designate other substances. Tremetol has the characters of an alcohol; the hydroxyls are not phenolic and alkoxy groups appear to be absent. It does not react with hydroxylamine or with phenylhydrazine to form crystalline substances, nor does it give color reactions with Schiff's reagent or with ferric chloride. It withstands the temperature of boiling water but is rapidly decomposed when attempts to distill it are made, even at pressures below 1 mm. It is very slowly volatile with steam. It is soluble in petroleum ether, chloroform, alcohol, and benzene, and quite insoluble in water, acids, or alkaline solutions.

It is levorotatory in alcoholic solution,  $[\alpha]_D^{20} = -21.08^\circ$ . It absorbs four atoms of bromine, which indicates the presence of two double bonds.

Potassium permanganate oxidizes it to a crystalline acid that melts at  $181^\circ C$ . Tremetol apparently exists in the plant partly in ester combination with a resinous acid, the chemistry of which is still being studied. The toxic substance may be prepared by the following method.

#### METHOD OF PREPARING TREMETOL

To obtain tremetol the fresh richweed plant is hashed through a meat chopper and immediately packed in a percolator with strong alcohol, macerated two days and then percolated to exhaustion with strong alcohol. The solvent is distilled from the percolate at ordinary pressure, leaving a watery residue mixed with a quantity of resin and fat. This is repeatedly extracted with boiling water to remove all water-soluble constituents. The water layers are siphoned off after each extraction. The final resinous residue is then extracted with boiling 50 per cent alcohol as long as soluble matter is obtained.

The alcoholic solutions are filtered off hot, united, and distilled to remove alcohol. The watery residue is allowed to cool and is filtered from precipitated resins. These resins are then extracted with boiling 30 per cent alcohol as long as they yield soluble matter. The solutions are filtered hot and the alcohol is removed from them. Crude tremetol ester separates out. This is purified by solution in alcohol and by boiling with 5 per cent alcoholic potash to hydrolyze the ester.

When hydrolysis is complete the alcohol is evaporated from the reaction product, and the residue is dissolved in water and extracted with ether, which dissolves the tremetol together with some coloring matters. The ether solution is poured into four volumes of petroleum ether, as a result of which process most of the coloring matters and other impurities are precipitated. Tremetol is recovered by evaporating the solvent, and should be purified by repeated solution in ether and precipitation with petroleum ether. The purity of the product may be determined by optical rotation and by ascertaining molecular weight. The absence of ash and deep color also indicates purity.

#### A COLOR REACTION OF TREMETOL

When a small quantity of tremetol is dissolved in petroleum ether and the solution is floated on the surface of concentrated sulphuric acid contained in a test tube, a red ring forms at the interface. If the two liquids are mixed by gentle agitation the petroleum ether is colored a transient cherry red which rapidly fades and the acid layer acquires a brilliant cherry-red color. If the solution is too dilute a yellow or orange may be the only result; if it is too concentrated or if much impurity is present the color reaction may be obscured by carbonization.

This reaction is offered as a provisional test for the presence of tremetol in *Eupatorium* mixtures. While there are numerous other substances which yield red colors with concentrated sulphuric acid, many of them are insoluble in petroleum ether and would not interfere with the test. Others give different shades of red. The cherry red given by tremetol is quite characteristic. A negative test definitely establishes the absence of tremetol; a positive reaction taken in conjunction with other data may demonstrate the presence of that substance.

A careful study of the applicability and specific nature of this test is in progress. It is hoped that through this color reaction a method may be developed by which suspected milk may be tested and dangerous milk detected, in areas where milk sickness exists.

It has been possible to use this test in order to check up on feeding experiments, particularly when failure to produce poisoning by extracts was not explicable. Unfortunately, samples of some of the extracts used during the course of this study were not available when the test was discovered and could not be tested. The results are stated in connection with the various discussions of the extracts.

#### COLLECTION OF PLANT MATERIAL

The plant material used in this investigation was collected in two localities in Illinois and in one place in Virginia, as shown in Table 1. The Illinois plant was gathered partly in the neighborhood of Beecher

City and partly at Paxton, in both of which localities milk sickness had appeared. The Virginia plant was collected on the banks of the Potomac River, Arlington County, in the neighborhood of Washington, D. C., a district from which no record of milk sickness has come. Both green and dried plants were studied. The dried plant originated wholly in Beecher City, Ill. One lot of green plant was collected at Beecher City, and was packed in milk cans, covered with chloroform water to preserve it, and shipped to Washington, D. C. Other lots of green material from Illinois were mailed to Washington where they arrived in fresh condition and were immediately hashed and put into strong alcohol. Green material was collected in Virginia and was carried to the laboratory within 24 hours. When used for direct feeding, the plant was usually collected early in the morning and was carried immediately to the laboratory. One lot of green plant, collected in Virginia by W. N. Berg, was immediately placed by him in strong alcohol and had been macerated for two years when it was worked up during the present study.<sup>3</sup>

TABLE 1.—*Collections of Eupatorium urticaefolium*

Lot No.	Date collected	Condition when used	Place of collection	Weight	Collector
E 1.....	Nov 17, 1916.....	Green.....	Arlington Co., Va.....	Kgm. 1.805	W. N. Berg.
E 2.....	October, 1915.....	Dry.....	Fancher (Beecher City), Ill.	4.432	A. B. Clawson.
E 3.....	Oct. 7-22, 1921.....	Green.....	Woodmont, Va.....	13.63	J. F. Couch.
E 4.....	Apr. 20-30, 1925.....	do.....	do.....	7.27	Do.
E 5.....	Sept. 22, 1920.....	do.....	Beecher City, Ill.....	28.0	Do.
E 6.....	Sept. 25-Oct. 3, 1921.....	do.....	Woodmont, Va.....	53.32	Do.
E 7.....	October, 1922.....	do.....	Beecher City and Paxton, Ill.	35.9	J. M. Casstevens and J. E. Parks.
E 8.....	October, 1915.....	Dry.....	Beecher City, Ill.....	18.93	A. B. Clawson.
E 9.....	October, 1923.....	Green.....	Beecher City and Paxton, Ill.	98.5	J. M. Casstevens and J. E. Parks.
E 10.....	September, 1925.....	do.....	Beecher City, Ill.....	131.8	J. M. Casstevens.

## ANIMALS USED IN THE EXPERIMENTAL WORK

The earlier experiments to test the toxicity of various extracts and their fractions were made on guinea pigs, but the variability of the results obtained with these animals and the impossibility of their exhibiting the symptoms characteristic of trembles in cattle led to abandonment of guinea pigs in favor of sheep. It was on the latter animal that all the crucial experiments were made. Cats were used in certain feeding tests, and rabbits were used in testing the toxicity of the resin acid.

All animals were allowed access to plenty of feed and water during the experiments. These experiments are summarized in Table 2.

<sup>3</sup> The writer desires to express his thanks to those who assisted in the collection of plant material, and especially to J. M. Casstevens and to J. E. Parks, of Illinois. Specimens of the various collections were submitted to S. F. Blake, botanist at the Bureau of Plant Industry, for identification before they were used in this investigation.

TABLE 2.—Summary of experiments in feeding *Eupatorium* extracts to animals

Date of feeding		Animal		Material fed			Dosage		Plant equivalent		Character of constituents	Effect	Termination
On or from—	To—	No.	Weight	Condition of plant	Origin	Fraction and laboratory No.	Quantity per single dose	Number of doses	Per single dose	Total			
1919	1919	Sheep	Pounds										
July 1		530	96	Dry	Fancher, Ill.	E 2. ABC	48.75 c. c.	4	3 lb.	12 lb.	Part of water solution	None	Survived.
2		539	96	do.	do.	E 2. ABC	73 c. c.	4	4 5 lb.	18 lb.	do.	do.	Do.
5		530	87	do.	do.	E 2. AAA	1 gm.	12	150 gm.	1,800 gm.	Resin acids	do.	Do.
8	July 8	530	87	do.	do.	E 2. AAA	2 gm.	4	300 gm.	1,200 gm.	do.	do.	Do.
9		530	85	do.	do.	E 2. AAA	3 gm.	7	450 gm.	3,150 gm.	do.	do.	Do.
11	July 10	530	85.25	do.	do.	E 2. AAA	4 gm.	4	600 gm.	2,400 gm.	do.	do.	Do.
12		530	85.25	do.	do.	E 2. AAA	5 gm.	4	750 gm.	3,000 gm.	do.	do.	Do.
13		530	81	do.	do.	E 2. AAA	15 gm.	2	2,250 gm.	4,500 gm.	do.	do.	Do.
15		522	86	do.	do.	E 2. AAB	150 c. c.	1	1,456 gm.		Lipoid fraction	do.	Do.
16		522	86	do.	do.	E 2. AAB	150 c. c.	1	2,912 gm.		Alkaloid	do.	Do.
Aug. 19		534	108.5	do.	do.	E 2. alkaloid		1	24 4 lb.		do.	do.	Do.
26	Sept 2	550	98	do.	do.	E 2. marc	214 gm.	4	214 gm.	856 gm.	Marc	do.	Do.
1920													
June 28		596	94.5	do.	do.	E 2. B	60 c. c.	1	3 lb.		Alcohol-insoluble, water solution.	do.	Do.
29	June 30	596	94.5	do.	do.	E 2. B	100 c. c.	3	5 lb.	15 lb.	do.	do.	Do.
July 1		596	94.5	do.	do.	E 2. B	150 c. c.	1	7 1/2 lb.		do.	do.	Do.
2		596	94.5	do.	do.	E 2. B	200 c. c.	2	10 lb.	20 lb.	do.	do.	Do.
1921													
July 6	July 19	639	86.5	Green.	Beecher City, Ill.	E 5. BAA	381 c. c.	14	2.5 lb. <sup>a</sup>	35 lb. <sup>a</sup>	Nonacidic, volatile	do.	Do.
16	July 31	650	72	do.	do.	E 5. BAB	162 c. c.	16	2.5 lb. <sup>a</sup>	40 lb. <sup>a</sup>	Nonvolatile, water soluble	do.	Do.
22	Aug. 4	622	85	do.	do.	E 5. AAA	50 c. c.	14	2.5 lb. <sup>a</sup>	35 lb. <sup>a</sup>	Resin acids	Pulse depressed	Do.
Aug. 2	Aug. 8	631	88	do.	do.	E 5. BAB	3.45 gm.	7	2.5 lb. <sup>a</sup>	17.5 lb. <sup>a</sup>	Volatile acids	None	Do.
30	Sept. 5	625	85.5	do.	do.	E 5. AAB	65 c. c.	7	2.5 lb. <sup>a</sup>	17.5 lb. <sup>a</sup>	Lipoid fraction	Trembles	Recovered
Sept. 6	Sept. 13	650	88	do.	do.	E 5. AAB	66 c. c.	7	2.5 lb. <sup>a</sup>	17.5 lb. <sup>a</sup>	do.	do.	Killed for autopsy.
1922													
July 10	July 21	677	98.75	do.	Woodmont, Va.	E 6.3 AB	8.7 gm.	14	2.5 lb. <sup>a</sup>	35 lb. <sup>a</sup>	Ligom solution of lipid fraction	Fever	Survived.
11	July 22	679	78.5	do.	do.	E 6.5 AC	0.872 gm.	12	2.5 lb. <sup>a</sup>	30 lb. <sup>a</sup>	Alcohol fraction of fats and resins.	None	Do.
15	July 25	674	73	do.	do.	E 6.4. AB	3.63 gm.	18	2.5 lb. <sup>a</sup>	40 lb. <sup>a</sup>	Benzene solution of lipoids,	do.	Do.

24	do.	679	78.5	do.	do.	E 6.5 AC	1.75 gm.	2	5 lb. <sup>a</sup>	10 lb. <sup>a</sup>	Alcohol fraction of fats and resins.	do.	Do.
24	do.	674	73	do.	do.	E 6.4 AB	7.26 gm.	2	5 lb. <sup>a</sup>	10 lb. <sup>a</sup>	Benzene solution of lipoids	do.	Do.
27	Aug. 1	679	78.5	do.	do.	E 6.5, AC and 4 AB.	13.3 gm.	6	2.5 lb. <sup>a</sup>	15 lb. <sup>a</sup>	Mixture of ligroin and benzene fraction.	do.	Do.
1923 Mar. 13	Mar. 16	707	62	do.	Beecher City, Ill.	E 7.3 A	25.9 gm.	4	4.6 lbs. <sup>a</sup>	18.4 lb. <sup>a</sup>	Fats and resin acids.	Trembles	Died Mar. 18.
Apr. 3	Apr. 11	708	82.5	do.	do.	E 7.3 AB	24.3 gm.	4	4 lb. <sup>a</sup>	16 lb. <sup>a</sup>	Fatty acids.	None	Survived.
Apr. 16	Apr. 18	708	67	do.	do.	E 7.4 A	10.22 gm.	3	4 lb. <sup>a</sup>	12 lb. <sup>a</sup>	Sterols	Trembles	Died.
16	Apr. 22	709		do.	do.	Milk of 708.						do.	Killed for autopsy.
July 3	July 7	779	38	do.	do.	E 7.4 AB	1.2 gm.	4	4 lb. <sup>a</sup>	16 lb. <sup>a</sup>	Phytosterol fraction.	None	Survived.
11	July 14	779	38	do.	do.	E 7.6 AII	1 gm.	4			Vacuum distillate No. 2.	do.	Do.
20	July 21	779	38	do.	do.	E 7.6 AII	1.5 gm.	2			do.	do.	Do.
25	July 26	779	38	do.	do.	E 7.6 A	2 gm.	4	5 lb.	20 lb.	Vacuum residue	do.	Do.
Aug. 2	Aug. 6	779	38	do.	do.	E 7.5 AB	1 gm.	4			Ligroin insoluble	do.	Do.
Aug. 14	Aug. 14	779	38	do.	do.	E 7.5 AB	3 gm.	1			do.	do.	Do.
20	Aug. 21	779	38	do.	do.	E 7.5 AII	1.5 gm.	2			Vacuum distillate No. 3.	do.	Do.
20	Aug. 23	779	38	do.	do.	E 7.6 AIII	0.46 gm.	4			do.	do.	Do.
28	Aug. 31	779	38	do.	do.	E 7.4 AB	2.5 gm.	4	8 lb. <sup>a</sup>	32 lb. <sup>a</sup>	Phytosterol fraction.	do.	Do.
Sept. 1		779	38	do.	do.	E 7.4 AB	2 gm.	1	6.4 lb. <sup>a</sup>	16 lb. <sup>a</sup>	do.	do.	Do.
4	Sept. 5	779	38	do.	do.	E 7.4 AB	2.5 gm.	2	8 lb. <sup>a</sup>	31.34 lb. <sup>a</sup>	Ligroin soluble	Symptoms	Do.
12	Sept. 13	779	38	do.	do.	E 7.5 A	3.5 gm.	3	15.67 lb. <sup>a</sup>		Vacuum distillate No. 1.	None	Recovered.
17	Sept. 19	779	38	do.	do.	E 7.6 AI	4.26 gm.	3			do.	do.	Survived.
26		779	38	do.	do.	E 7.	5 gm.	1	12.6 lb.	33.1 lb. <sup>a</sup>	Volatile oil.	Not observed	Died.
1924 Jan. 2	1924 Jan. 3	782	79	Dry	do.	E 8. B	1.25 gm.	4			Vacuum distillates.	None	Survived.
Apr. 16	Apr. 30	782	105	Green	do.	E 7.	1 gm.	13	2.52 lb.	32.75 lbs <sup>a</sup>	Volatile oil.	do.	Do.
June 9	June 12	782	105	do.	do.	E 9. AAA	2 gm.	4	4.4 lb. <sup>a</sup>	17.6 lb. <sup>a</sup>	Alcohol-insoluble oil from sterol fraction.	do.	Do.
18	June 21	782	105	do.	do.	E 9. AAC	0.5 gm.	4			Steam distillate.	do.	Do.
24	June 25	782	105	do.	do.	E 9. AAD	2.9 gm.	2	5.5 lb.	11 lb.	Sterol residue from steam distillate.	do.	Do.
26	June 27	782	105	do.	do.	E 9. AAD	2.3 gm.	2	4.4 lb.	8.8 lb.	do.	do.	Do.
30	July 5	782	105	do.	do.	E 9. AAD	4.6 gm.	5	8.8 lb.	44 lb.	do.	do.	Do.
July 15	July 18	782	105	do.	do.	E 9. AAA	3.75 gm.	4	7.15 lb.	28.6 lb.	Oil from sterol fraction.	do.	Do.

<sup>a</sup> Per hundredweight of animal.

TABLE 2.—Summary of experiments in feeding *Eupatorium* extracts to animals—Continued

Date of feeding		Animal		Maternal feed			Dosage		Plant equivalent		Character of constituents	Effect	Termination
On or from—	To—	No.	Weight	Condition of plant	Origin	Fraction and laboratory No.	Quantity per single dose	Number of doses	Per single dose	Total			
1925 Feb. 9	1925 Feb. 12	Sheep 782	Pounds	Dry	Beecher City, Ill.	E 9. A	27.4 gm.	4	4 lb. <sup>a</sup>	16 lb. <sup>a</sup>	Alcohol extract	None	Survived.
Apr. 1	Apr. 9	4	57	do	do	E 9. A	15 gm.	8	4 lb. <sup>a</sup>	32 lb. <sup>a</sup>	do	do	Do.
Apr. 22	Apr. 25	4	60	do	do	E 9. A	30 gm.	4	8 lb. <sup>a</sup>	32 lb. <sup>a</sup>	do	do	Do.
June 20	June 30	5	75	do	Woodmont, Va.	E 4	1.66 lb. <sup>a</sup>	9	1.66 lb. <sup>a</sup>	15 lb. <sup>a</sup>	Green plant	do	Do.
1926 Mar. 4	1926 Mar. 6	5	128	do	Beecher City, Ill.	E 10. AAB	7 gm.	3	1.65 lb. <sup>a</sup>	5 lb. <sup>a</sup>	Solution in 50 per cent alcohol.	do	Do.
Mar. 18	Mar. 18	5	128	do	do	E 10. AAB	10.56 gm.	9	2.5 lb. <sup>a</sup>	22.5 lb. <sup>a</sup>	Insoluble in 50 per cent alcohol.	do	Do.
Apr. 3	Apr. 3	5	123	do	do	E 10. AAA	27 gm.	12	3 lb. <sup>a</sup>	36 lb. <sup>a</sup>	do	do	Do.
Apr. 12	Apr. 15	5	123	do	do	E 10. AAB	21.12 gm.	4	5 lb. <sup>a</sup>	20 lb. <sup>a</sup>	Soluble in 50 per cent alcohol.	Trembles	Died.
May 8	May 13	6	69	do	do	E 10. AAB		5	7.5 lb. <sup>a</sup>	37.5 lb. <sup>a</sup>	Insoluble in 30 per cent alcohol.	None	Survived.
Aug. 3	Aug. 10	6	70	do	do	E 7. AAB	1.95 gm.	7	7.5 lb. <sup>a</sup>	52.5 lb. <sup>a</sup>	Resin acid.	do	Do.
Aug. 20	Aug. 25	6	75	do	do	E 7. B	100 gm.	5	8 lb. <sup>a</sup>	40 lb. <sup>a</sup>	Water soluble.	do	Do.
1918 Nov. 20	1918 Nov. 18	Guinea pig 52	Grams	Green	Arlington County, Va.	E 1. AB	0.08 gm.	10	10 gm.	100 gm.	Volatile oils and fats.	None	Do.
Nov. 20	Nov. 27	53	365	do	do	E 1. AB	0.08 gm.	10	10 gm.	100 gm.	do	do	Do.
Nov. 21	Nov. 27	46	560	do	do	E 1. ACB	13.13 gm.	5	65.6 gm.	328.3 gm.	Water soluble.	do	Killed for autopsy.
Nov. 29		55	305	do	do	E 1. AA		1	900 gm.		Resin	Sick	Died Nov. 30.
Dec. 13	Dec. 19	57	310	do	do	E 1. AAB		6	150 gm.	900 gm.	Soda solution of resin	do	Died Dec. 31.
1919 Jan. 2	1919 Jan. 2	70	535	do	do	E 1. AAA		1	900 gm.		Resin insoluble in soda	do	Died Jan. 15.
1921 Oct. 7	1921 Oct. 22	192	670	do	Woodmont, Va.	E 3. fresh leaves.		10	21.75 gm.	217.5 gm.	Total	do	Survived.
Dec. 7	Dec. 19	193	590	do	do	E 6.3 AB	1 gm.	10	21.75 gm.	217.5 gm.	do	do	Died Nov. 9.
Dec. 13	Dec. 19	197	560	do	do			3	130 gm.	390 gm.	Ligroin solution	do	Recovered.

[illegible]

**Meat of sheep fed.**

Per hundred weight of animal.

## EXTRACTING AND FRACTIONATING THE PLANT

Eight of the lots of plant collected for use in this study were submitted to extraction and fractionation processes, and inasmuch as the procedure was similar in every case a composite description of the procedure followed is presented here, the various fractions being distinguished by a lot number in order to insure clearness. The fractions are further grouped according to the chemical character of the substances present so that comparison may be made between various fractions and between plants from different collections.

In general, the fresh plants were subjected to the following process to extract the soluble constituents and to fractionate the extracts so obtained: The fresh plant was hashed and placed in strong alcohol in a percolator. After two days' maceration the plant was extracted with alcohol by intermittent percolation; the alcohol was removed from the percolate by distillation at atmospheric pressure and was returned to the percolator. The residue from the distillation was mixed with water and redistilled to remove the last traces of alcohol and also to drive off the volatile oil which was recovered.

The residue so obtained consisted of a greenish, insoluble mass of lardy consistency, and a watery solution. The water solution was siphoned off, and the insoluble residue was repeatedly extracted with boiling water as long as it yielded soluble matter. The combined water extracts furnished one fraction.

The insoluble matter was next treated with hot 1 per cent sodium-carbonate solution which dissolved out a resin acid fraction, leaving undissolved what was termed the "lipoid" fraction. The latter was fractionated in various ways, which are described in connection with the testing of the fractions themselves.

The water-soluble materials were fed in aqueous solution. The resin acid was fed in sodium-carbonate solution, and other materials were emulsified in water or in sirup by means of acacia.

## FRESH LEAVES

Leaves from plants collected at Woodmont, Va., were fed to two guinea pigs October 7 to 22, 1921, a total of 10 doses, averaging 21.75 gm. per dose, being given. The animals had access to oats during the experiment. Both were made sick, and one died 18 days after the last feeding. Post-mortem examination showed liver and kidneys pale, lungs congested, and congestion in the stomach and colon. The other animal survived.

Sheep 5 was fed by balling gun nine doses of fresh leaves collected at Woodmont, Va. From June 20 to 30, 1925, this sheep was fed 5.123 gm. of fresh plant, equivalent to 15 pounds per hundred-weight of animal. This feeding produced no effect on the animal.

## TOTAL WATER-SOLUBLE NONVOLATILE CONSTITUENTS

The material of plant lot E 5, collected in Beecher City, Ill., and shipped to Washington preserved in chloroform water, was allowed to stand in storage during the winter. It was opened in May, 1921, when the water solution was strained off. The leaves of the plant were stripped from the stems, hashed in a meat chopper and pressed out, the strained juice being added to the water solution. Half of

the solution was distilled from a copper still until volatile substance, other than water ceased to appear in the distillate. The nonvolatile residue (E 5. BAB) was collected and used for a feeding test. This was a limpid, faintly greenish-brown, acid liquid, of bitter, saline, and highly unpleasant taste. It was colored deep green with ferric chloride, even after the removal of possible tannins with gelatine solution. It yielded no alkaloid test with Mayer's solution. Alcohol precipitated a large amount of gray, flocculent matter (inulin). The whole residue was concentrated to 2,700 c. c. by evaporation.

Sixteen doses of 162 c. c. of this solution, corresponding to 2.5 pounds of fresh plant per hundredweight of animal, were drenched into sheep 650 from July 16 to July 31, 1921. The total dosage was 2,592 c. c., equivalent to 40 pounds of plant per hundredweight. The sheep was not affected by this treatment.

#### VOLATILE OIL

Volatile oils were obtained in concentrating alcoholic extracts of *Eupatorium*. Oils obtained from green plant gave a positive test for tremetol; a sample from dry plant was negative. An emulsion containing 5 gm. of oil obtained from lot E 7 was drenched into sheep 779 September 25, 1923, at 1 p. m. The animal was observed during the afternoon but showed nothing abnormal. It was found dead, however, on the following day at 6.30 a. m.

A post-mortem examination conducted during the morning by G. T. Creech, of the pathological division, revealed hemorrhagic areas on the endocardium; the liver pale and degenerate; the kidneys degenerate; hemorrhages in the lower end of ileum and duodenum; and thyroid and thymus congested. There was much fluid in the abdominal cavity. Certain of these abdominal conditions were considered to be of longer standing than the present feeding would account for, and possibly were the result of earlier experiments made on this sheep.

The volatile oil, emulsified in sirup with acacia, was fed to cats 17 and 19. On November 26, 1923, cat 17 received 0.45 gm. of oil and November 27, 0.60 gm. On November 26 cat 19 received 0.30 gm. and November 27, 0.60 gm. of oil. Both animals survived the doses and showed no abnormal effects from them.

From April 15 to 30, 1924, sheep 782 was given 13 daily doses of 1 gm. each of oil, emulsified in sirup with acacia. The total dosage, equivalent to 32.75 pounds of green plant per hundredweight of animal, produced no effect.

From these experiments it is apparent that the essential oil, while toxic in large doses, is not the constituent responsible for trembles.

#### WATER- AND ALCOHOL-SOLUBLE CONSTITUENTS

##### LOT NO. E 1

The material consisted of 1,805 gm. of green plant that had been hashed and put into alcohol November 17, 1916, by W. N. Berg, then of this laboratory, who turned the specimen over to the writer in 1918. October 15, 1918, it was packed into two glass percolators and exhausted with strong alcohol. The alcohol was removed from the percolate by distillation, leaving a brown, aqueous solution on

the surface of which was an oily layer. A resinous precipitate was distributed throughout the aqueous solution. The whole was extracted with petroleum ether (E 1. AB). The water residue was filtered from the precipitated resin and tested on guinea pig 46, in 10 c. c. doses, equivalent to 65.64 gm. of green plant. Five doses were given from November 21 to November 27. The animal showed no abnormal effects from these doses. It was killed for autopsy, which revealed a slight gastroenteritis.

LOT NO. E 2

Four thousand four hundred and thirty-two gm. of dried whole plant were ground to a No. 20 powder and extracted with strong alcohol. The solvent was distilled from the percolate and the resinous residue extracted with boiling water. The filtered water solution was evaporated to convenient bulk. It responded to Mayer's test for alkaloids; consequently it was made alkaline with sodium hydroxide and shaken out first with chloroform which removed very little, and then with amyl alcohol which removed a basic substance.

VOLATILE, WATER-SOLUBLE MATERIAL

The distillate from the water solution of E 5 was cohobated until its volume was reduced to 6 liters. It was treated with a slight excess of sodium hydroxide and submitted to steam distillation until pure water alone came over. The distillate (E 5. BAAA) bore the characteristic odor of the plant, but only minute quantities of oil separated. It did not react with ferric chloride. Fourteen doses of it drenched into sheep 639 resulted in no other effect than a slight catharsis. The alkaline residue (E 5. BAAB) was evaporated to dryness when it weighed 48 gm. Half of it was dissolved in water, neutralized with acetic acid, and fed to sheep 631 in divided doses. The sheep remained normal.

The extracted water solution (E 2. ABC) was fed to sheep 539. Four doses estimated to represent 3 pounds of green plant each and 4 doses estimated to represent 4.5 pounds of green plant each, a total of 30 pounds, fed to this animal, produced no ill effects.

LOT NO. E 5

The press cake (see p. 560) was extracted similarly with alcohol. The resinous residue obtained was fractionated with sodium-carbonate solution which dissolved the remaining water-soluble constituents as well as a resin acid. The testing of this fraction is described under the heading "Resin acids."

LOTS NOS. E 6, E 7, E 9, AND E 10

E 6, E 7, E 9, and E 10 yielded similar water solutions. E 6 B was fed to sheep 6 in 100 gm. doses. Five daily doses each, equivalent to 8 pounds per hundredweight given to the animals, produced no effect. The water solution gave evidence of the presence of a glucoside and responded to Mayer's test. Acetic acid was present, and levulose, probably resulting from the hydrolysis of inulin, was detected. The water solution from E 7 was tested for tremetol with negative results.

## ALKALOID

The amyl-alcohol solution containing the alkaloidal substance extracted from E 2. ABC was treated with dilute hydrochloric acid which extracted the base. A quantity of this, estimated to be equivalent to 24.4 pounds of green plant, was fed to sheep 534 August 19, 1919, and did not affect the animal.

## ALCOHOL-SOLUBLE, WATER-INSOLUBLE CONSTITUENTS

## FATS, RESINS, RESIN ACIDS, ESTERS

## LOT NO. E 1.

Half of the resinous precipitate was emulsified in mucilage or Irish moss and given to guinea pig 55 in a dose equivalent to 900 gm. of the green plant. The animal died during the night after showing symptoms of depression. The autopsy was negative.

Thereafter all resinous fractions were treated with sodium-carbonate solution before testing on experimental animals.

The remaining half of the resinous fraction was treated with sodium carbonate solution which dissolved a large portion of it. This fraction was given to guinea pig 57 in doses beginning December 13, 1918. The animal became depressed, rapidly lost weight and died on December 31 showing symptoms of respiratory paralysis. An autopsy showed the animal greatly emaciated; the organs, however, appeared normal.

## LOT NO. E 2

A similar fraction containing resin acids (E 2. AAA) was dissolved in sodium-carbonate solution and fed to sheep 550 in 33 doses from July 5 to July 13, 1919. The quantity fed was increased from 1 to 15 gm. per dose. An equivalent of 35.31 pounds of green plant fed to the sheep during this period produced no effect.

## LOT NO. E 5

The resin-acid fraction from this extraction (E 5. AAA) was dissolved in sodium-carbonate solution and fed to sheep 622 from July 22 to August 4, 1921, in doses equivalent to 2.5 pounds per hundred-weight. Fourteen doses were administered, equal to 35 pounds of green plant. There was a slight but definite depression of the pulse but no other abnormal symptom was observed and the animal recovered.

## LOT NO. E 7

The homologous fraction (E 7. AAB) was fed to sheep 6 from August 3 to August 10, 1926, in seven daily doses, each equivalent to 7.5 pounds of green plant per hundredweight of animal, or a total of 52.5 pounds. There was no effect from this feeding.

A series of feedings of this fraction were made to 6 guinea pigs and 2 rabbits. The results are tabulated in Tables 3 and 4. Of the 6 guinea pigs, 5 were made sick and 4 of them died.

TABLE 3.—Quantities of resin acids fed to rabbits and guinea pigs, 1926

Date	Guinea pig No.						Rabbit No.	
	297	298	299	300	301	302	28	29
	<i>Mgm.</i>	<i>Mgm.</i>	<i>Mgm.</i>	<i>Mgm.</i>	<i>Mgm.</i>	<i>Mgm.</i>	<i>Mgm.</i>	<i>Mgm.</i>
Aug. 9.....	39	78	136.5	58.5	117	156	476.15	238.07
Aug. 11.....	39	78	136.5	58.5	117		476.15	238.07
Aug. 20.....	78		273	117	234		952.3	476.15
Aug. 28.....			273	117			952.3	476.15
Sept. 2.....			273	117				

TABLE 4.—Weights of guinea pigs and rabbits fed resin acids, 1926-27

Date	Guinea pig No.						Rabbit No.	
	297	298	299	300	301	302	28	29
	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>
1926:								
Aug. 9.....	460	430	545	470	510	560	1,865	1,635
Aug. 11.....	480	450	550	490	510	Dead.	1,845	1,595
Aug. 14.....	470	400	580	495	500		1,815	1,595
Aug. 16.....	435	355	575	495	500		1,880	1,605
Aug. 19.....	400	Dead.	595	525	520		1,870	1,610
Aug. 26.....	300		625	545	Dead.		1,895	1,630
Aug. 28.....	310		625	540			1,895	1,605
Sept. 2.....	Dead.		640	525			1,880	1,670
Sept. 29.....							1,550	1,790
Oct. 7.....							1,380	1,740
Oct. 11.....							1,305	1,715
Oct. 16.....							1,115	1,690
1927:								
Jan. 7.....								1,450

Guinea pig 302 may have succumbed to a traumatic pneumonia. The three other pigs remaining, which died from the dosage, all showed the same symptoms of depression. The autopsies revealed anemia, great emaciation, enlarged liver and spleen. The urine collected at autopsy was usually pale, cloudy, acid, and contained albumin. Acetone, dicetic acid, and reducing substances were not found. The two rabbits fed were made sick. Rabbit 28 died October 17, after losing a total of 750 gm. of its original weight. The post-mortem examination revealed emaciation and anemia. Urine collected at autopsy was acid and contained albumin. Sugar and acetone bodies were absent.

Rabbit 29 was observed for five months and was finally discharged January 7, 1927, when he had lost a total of 185 gm. of his original weight. This animal survived the treatment, but appeared to be permanently affected, inasmuch as he failed to gain weight or to recover from depression, although more than four months had elapsed since the last feeding of resin acids.

#### NONACIDIC CONSTITUENTS OF THE RESINOUS FRACTION

#### FATS, WAXES, ESTERS, AND "LIPOID FRACTION"

#### LOT NO. E 1

The portion of the resin not soluble in sodium carbonate was emulsified in 50 per cent alcohol and given to guinea pig 70 January 2, 1919. The animal became depressed, lost 26 per cent of his

original weight, and finally died January 14. Post-mortem change was so far advanced that no conclusions could be drawn from the autopsy.

## LOT NO. E 2

A similar fraction (E 2. AAB) was emulsified in water and drenched into sheep 522 in two doses. No effect was observed.

## LOT NO. E 5

The corresponding fraction (E 5. AAB) was emulsified in water with acacia and seven doses of 65 c. c. each, equivalent to 2.5 pounds of green plant per hundredweight of animal, were given to sheep 625 from August 30 to September 5, 1921. The sheep developed a positive case of trembles. Feeding was discontinued and the animal slowly recovered. Another portion of the same fraction was given in seven doses of 66 c. c. (2.5 pounds per hundredweight) to sheep 650 from September 6 to September 13. The animal developed a positive case of trembles. She was killed for autopsy September 19. The post-mortem examination showed some slight congestion in the lower ileum, liver pale and yellowish, and the cortex of the kidney dark. A sample of blood drawn from the left jugular vein immediately after death was tested for acetone bodies, with a positive result.

Specimens of urine obtained from both sheep 625 and sheep 650 contained acetone, and the odor of that ketone was noticeable on the exhaled air in both cases.

## LOT NO. E 6

The corresponding fraction from this extraction (E 6. AAA) was not fed as such but was divided by means of solvents into several portions, as follows:

Petroleum ether extracted---	0.768 per cent of green plant (E 6. 3AB)
Benzene extracted-----	0.36 per cent of green plant (E 6. 4AB)
Alcohol extracted-----	0.098 per cent of green plant (E 6. 5AC)

The alcohol extract was divided, and a small portion of it was extracted successively with ether and chloroform, as follows:

Ether extracted-----	0.062 per cent of green plant (E 6. 5AB)
Chloroform extracted-----	0.033 per cent of green plant (E 6. 6AB)

The material extracted by the solvents was given to sheep and guinea pigs.

The petroleum-ether fraction was emulsified in water with acacia and 12 doses, each equivalent to 2.5 pounds of green plant per hundredweight of animal, were drenched into sheep 677 from July 10 to July 21, 1922, with no effect on the animal. Three doses of 1 gm. each (emulsified) were given to guinea pig 197 from December 13 to December 19, 1921. The animal was made sick but recovered.

A series of 21 doses of the fraction emulsified in water with acacia were given to guinea pigs 199 and 200 from March 8 to April 1, 1922, the doses being gradually increased from 2 to 5 gm. during the interval. Guinea pig 200 was made sick and died April 9. Pig 199 showed no abnormal effect from the feeding and survived. Guinea pig 197 which received 3 gm. doses of this fraction from December 13 to December 19, 1921, was made sick, but eventually recovered. This fraction gave a faintly positive test for tremetol.

The benzene fraction was emulsified in water with acacia and fed to guinea pigs 199 and 200 from January 26 to February 16, 1922. Each animal received 14 doses of 0.3 gm. corresponding to 78.3 gm. of green plant, or a total of 1,096.2 gm. of green plant for the entire period. Neither animal was affected by this treatment; both gained weight and appeared perfectly healthy during the experiment. The fraction was emulsified and fed to sheep 674 from July 15 to July 25, 1922, in eight doses of 2.5 pounds and two doses of 5 pounds of green plant per hundredweight. The total dosage, 30 pounds, did not affect the animal. This fraction did not respond to the test for tremetol.

The ether fraction was emulsified in water with acacia and fed to guinea pigs 201 and 202. Each animal received seven daily doses of 0.1 gm., equivalent in all to 200.7 gm. of green plant, from February 20 to February 28, 1922. Neither animal was affected by the material given.

The alcohol fraction was emulsified in water with acacia and fed to sheep 679 in 12 daily doses of 2.5 pounds per hundredweight from July 11 to July 22, 1922. On July 24 and 25 the doses were doubled so that the sheep received a total of 40 pounds per hundredweight. The sheep showed some slight symptoms of colic on July 22 and 23 but otherwise remained normal. The alcohol fraction was also fed to guinea pigs 201 and 202 in four doses totaling 0.75 gm. of fraction, equivalent to 1,405 gm. of green plant. Sheep No. 202 was not affected but sheep No. 201 became sick and died one month after the last feeding. The post-mortem examination revealed anemia, a pale liver, and pale kidneys. The fraction did not respond to the test for tremetol.

A mixture of the petroleum-ether and benzene fractions was made in equivalent proportions, and this mixture, equal to 15 pounds of green plant per hundredweight of sheep, was emulsified in water with acacia and fed to sheep 679 from July 27 to August 1, 1922, in immediate succession to feedings with the alcohol extract. (See above.) There was no effect from this treatment.

#### LOT NO. E 7

The residue obtained by distilling the solvent from the alcoholic percolate of this plant consisted of mixed fats, resins, and water solution. It was so difficult to filter that it was extracted with benzene, which dissolved the major portion of the water-insoluble matter present. The benzene solution was washed with several portions of aqueous sodium carbonate to free it from acidic constituents, separated from the water solution, and the benzene distilled off. The residue was a deep-green mass of the consistency of lard. It weighed 664 gm. and constituted 2.04 per cent of the green plant extracted. Four daily doses of an emulsion of this fraction were given in drenches to sheep 707. The animal received a total of 18.4 pounds of green plant equivalent per hundredweight from March 13 to March 16, 1923. The animal developed trembles and died during the night of March 18. The post-mortem examination revealed the lungs congested, light-colored areas in the liver which had the appearance of fatty degeneration, and the spleen pale. The other organs and the musculature were pale. Post-mortem change was marked. The

meat from this carcass was fed to cats 17, 18, and 19 from March 19 to March 28, 1923, but did not affect them.

Two doses of this fraction were given to guinea pig 213, the first February 28 and the second March 1, 1923. Each dose consisted of 0.2 gm. of the fraction and was equivalent to 10 gm. of green plant. The animal remained normal until March 10, when symptoms of depression appeared. These became progressively worse and the animal died March 23. The autopsy showed some congestion of the lungs, spleen pale and brownish, liver dark, and other organs normal. Urine taken from the bladder did not contain acetone.

LOT NO. E 9

A similar fraction (E 9. A) was emulsified in sirup with acacia and four doses of 27.4 gm., equivalent in all to 16 pounds of green plant per hundredweight, were given to sheep 782 from February 9 to February 12, 1925, without affecting the animal. The same fraction was fed twice to sheep 4 without producing trembles; from April 1 to April 9, 1925, this sheep received eight daily doses of 15 gm., a total of 32 pounds of green plant per hundredweight, and from April 22 to April 25, 1925, the sheep received four daily doses of 30 gm., equivalent in all to 32 pounds of green plant per hundredweight. Various fractions of this extract were also fed and are described below. In no case were symptoms observed and it was concluded that the original plant was nontoxic or that the process of extracting the soluble constituents had in some way altered the toxic principle.

Inasmuch as E 7 was processed in the same way and was toxic, it does not seem probable that the last hypothesis is correct. This fraction was later tested for tremetol and was found to contain a very small quantity of the toxic substance.

LOT NO. E 10

The corresponding fraction from this lot was obtained by treating the residue that remained, after the alcohol had been distilled from the percolate, with boiling water as long as soluble material was extracted. The insoluble material was then extracted with boiling 50 per cent alcohol as long as anything could be extracted. The insoluble matter was labeled E 10. AAA. It gave no test for tremetol. Twelve daily doses of this fraction, emulsified in sirup with acacia, and consisting of 27 gm. each, equivalent to 3 pounds of green plant per hundredweight, were given to sheep 5 from March 22 to April 3, 1926. The sheep was not affected. The material dissolved in 50 per cent alcohol was recovered by distilling off the solvent. This fraction responded to the test for tremetol. It was emulsified in sirup with acacia and given to sheep 5. From March 4 to March 6, 1926, the sheep received three daily doses of 7 gm., equivalent to 1.65 pounds of green plant per hundredweight. From March 7 to March 18, 1926, the sheep received nine daily doses of 10.56 gm., equivalent to 2.5 pounds per hundredweight. No effect was apparent, although the total dosage was equivalent to 27.5 pounds of green plant.

From April 12 to April 15, 1926, the same sheep was given four daily doses of 21.12 gm. each, corresponding to 5 pounds of green plant per hundredweight, or a total of 20 pounds. The sheep remained normal until April 16, when he began to tremble. He became pro-

gressively worse and died April 22. During the sickness several samples of urine and blood were obtained. On April 16, when trembling first appeared, the sheep was not excreting acetone bodies, but on the following morning there was an acetone odor on the breath and the urine contained a quantity of this ketone. From that day onward the animal excreted acetone continuously by the lungs and kidneys. At autopsy the only marked abnormality was apparent degeneration of the liver. The carcass had a strong odor of acetone. Blood and urine, collected at autopsy, were tested and found to contain acetone. In the urine the proportion of acetone was 24.32 mg. per 100 c. c. The blood sugar was markedly high during the sickness. On March 22, when the sheep appeared to be normal, a sample of blood drawn from the left jugular vein was subjected to analysis and the content of blood sugar was determined as 0.104 gm. per 100 c. c. Blood drawn similarly April 16 when the sheep was trembling gave an average of 0.168 gm. per 100 c. c. for two determinations. At this time feeding with the Eupatorium extract was discontinued and the sheep was given oats. Another sample of blood was taken April 22 from which a blood sugar content of 0.138 gm. per 100 c. c. was determined.

The remainder of the extract that had proved fatal to sheep 5 was treated with boiling 30 per cent alcohol in successive portions as long as soluble matter was dissolved. The insoluble residue (E 10. AABB) which gave no test for tremetol, was tested on sheep 6. Five daily doses of the emulsified material, equivalent to 7.5 pounds of green plant per hundredweight of sheep per dose, were given from May 8 to May 13, 1926. The total dosage was 37.5 pounds. There was no effect from this feeding.

The results obtained from feeding experiments with the "lipoid" fraction indicated clearly that the toxic principle responsible for trembles was contained in that fraction. Failure to produce poisoning in sheep with the extracts made from lots E 6 and E 9 was apparently due to their small content of tremetol. Lot E 6 was collected at Woodmont, Va., in a district where milk sickness had not been observed, so far as the writer knows. It is possible that plants from this locality are innocuous. Lot E 9, however, was collected in two places in Illinois from which very toxic plants were obtained at other times. Lot E 2 failed to yield a toxic lipid fraction; this material was dried plant, however, and was not expected to be very toxic.

#### HYDROLYSIS OF THE LIPOID FRACTION

##### LOT NO. E 7

Failure to obtain crystalline substances by chemical treatment of the fraction led to the decision to hydrolyze it with alcoholic potassium hydroxide. Accordingly this was done. A quantity of the toxic fraction was boiled under reflux with 5 per cent alcoholic potash until hydrolysis was complete; the alcohol was then removed and the bright-green residue was treated with water in which the soaps dissolved and emulsified the insoluble matter. This mixture was then repeatedly extracted with ether, which slowly removed a yellowish mass that was obtained by evaporating the solvent. The residue is described below as the "sterol" fraction. The soaps were decomposed with hydrochloric acid, and the precipitated mass was filtered off. The tests made on this material are described under the heading

"fatty acids." The filtrate was further extracted with ether to recover the water-soluble acids; the ether was shaken with sodium-carbonate solution, which took out a small quantity of acetic acid. Following the ether extraction the water solution was evaporated to small volume, and glycerine was detected in the residue.

#### THE FATTY ACIDS

LOT NO. E 7

The precipitate containing the fatty acids was washed with water, and a portion was emulsified in acacia and water for feeding. From April 3 to April 11, 1923, sheep 708 received four doses of this emulsion, each equivalent to 4 pounds of green plant per hundredweight, or a total of 24.3 gm. of the fraction.

On April 4 the sheep gave birth to a normal lamb. The animal was rested for 5 days before the experiment was resumed. There was no toxic effect from this feeding.

#### THE STEROL FRACTION

LOT NO. E 7

The material extracted by ether from the mixture of hydrolytic products consisted of a yellowish, partly crystalline, unctuous mass which had an agreeable aromatic odor, somewhat resembling that of nutmeg. Three daily doses of this fraction each containing 10.72 gm., equivalent to 4 pounds per hundredweight, were fed in emulsion to sheep 708 from April 16 to April 18, 1923. On April 19 the animal was unable to stand without trembling. The symptoms of trembles became worse, and the animal died Sunday morning, April 22. By the following day post-mortem change was so advanced that no conclusions could be drawn from autopsy.

The suckling lamb of this ewe, sheep 709, had received the milk from the mother during the feeding and the subsequent disease period. At the beginning of the experiment the lamb was healthy and normal. On April 21, two days after the appearance of trembles in the mother, the lamb also showed symptoms of trembles. These developed rapidly until the lamb was unable to stand. After the death of the mother the lamb was fed cow's milk for several days. During this period no improvement occurred. A specimen of urine was obtained April 26. This was acid and contained acetone. The lamb was also excreting acetone by the lungs. The lamb was killed for autopsy April 28 and the post-mortem examination was immediately made. The liver appeared discolored and the kidneys were degenerated. The other organs appeared to be normal.

This experiment definitely located the toxic principle in the sterol fraction and there arose the problem of separating it from the other sterols present. A portion was submitted to steam distillation, but only a very small quantity of volatile material was obtained. The main portion of the sterol fraction was dissolved in hot 85 per cent alcohol and allowed to cool when a considerable quantity of needle-shaped crystals separated out. These were collected by filtration and the filtrate was twice concentrated, each time yielding a crop of the same substance. These crops were united and recrystallized from 85 per cent alcohol, when the substance was obtained pure.

The mother liquors yielded an impure crop of this substance that was contaminated with oily matter.

The mother liquors from which the crystalline sterol was obtained were concentrated by evaporation, but nothing further could be made to crystallize from them. The solvent was removed and left a viscous, oily residue of aromatic odor. The larger portion was soluble in petroleum ether. The soluble matter was labeled E 7.6 A, the insoluble E 7.5 AB.

#### THE CRYSTALLINE STEROL

The crystalline sterol was a mass of soft, white needles melting at 148 to 149° C. and soluble in the ordinary organic solvents. Elementary analysis indicates that the formula is  $C_{18}H_{30}O$ . It does not give the characteristic reactions of phytosterols. A quantity of this substance was emulsified in water with acacia. Four daily doses each containing 1.2 gm. of the substance, equivalent to 4 pounds of green plant per hundredweight of sheep, were fed to sheep 779 from July 3 to July 7, 1923, but produced no effect on the animal. A second feeding of this mixture was made between August 28 and September 5, 1923, when the same sheep received 6 daily doses of 2.5 gm. and one of 2 gm., or a total of 54.4 pounds equivalent of green plant. There was no effect from these feedings.

#### LOT NO. E 9

A similar fraction from another lot (E 9. AAD), which consisted largely of the sterol, was fed in varying doses from June 24 to July 5, 1924, to sheep 782. This animal received in all 9 doses containing a total of 33.4 gm. of the fraction, equivalent to 63.8 pounds of green plant per hundredweight. There was no effect from this feeding.

These findings showed that the toxic principle was to be sought for in the other two fractions of the original sterol fraction, the portion soluble in petroleum ether, and the small quantity of material insoluble in that solvent.

#### THE PETROLEUM-ETHER INSOLUBLE FRACTION

This fraction labeled E 7.5 AB was a thick, brownish noncrystalline mass which weighed 8 gm. Nothing crystalline could be prepared from it. It was emulsified in water with acacia and fed in four doses of 1 gm. each to sheep 779 from August 2 to August 6, 1923, and in a dose of 3 gm. August 14. There was no effect from the feeding.

#### THE PETROLEUM-ETHER SOLUBLE FRACTION

The experimental feedings, described above, which showed the lack of toxicity of all other fractions of the original sterol fraction made it evident that E 7.6 A contained the toxic principle. Efforts were then directed toward separating this fraction into its constituents, which were mainly tremetol, with some crystalline sterol, coloring matters, probably carotin, and perhaps other unknown substances. A portion of the crystalline sterol was removed by cooling the fraction to a low temperature and filtering it from the separated crystals. Other methods of separation were tried but none were successful, and finally it was decided to distill the fraction under very diminished pressure and to test the distillates.

Three fractions were obtained, upon vacuum distillation, as follows:

Distillate I, 188 to 192° C. (15 mm.) 15 gm.

Distillate II, 192 to 256° C. 10 gm.

Residue.

The distillates were united and again distilled. Four fractions were obtained as follows:

Distillate I, 166 to 170° C. (15mm.) 5 gm. Tremetol test, negative.

Distillate II, 170 to 205° C. (12 mm.) 10 gm. Tremetol test, positive.

Distillate III, 205 to 235° C. (12 mm.) 5 gm. Tremetol test, negative.

Residue very small.

The temperatures at which the second distillates were obtained indicate that there had been some decomposition during the first distillation.

All the distillates were thick, oily liquids; the higher boiling fraction on cooling deposited a small quantity of crystals.

Distillate II was injected intraperitoneally into three guinea pigs June 14, 1923. Pig No. 236 received 0.2 c. c., pig No. 211 received 0.3 c. c., and pig No. 239 received 0.5 c. c. All were made sick and No. 239 died during the night of June 15-16. The post-mortem examination revealed a considerable yellowing of the liver and pale kidneys, the other organs being normal. Guinea pigs 211 and 236 were depressed for several days but finally recovered.

Four grams of this fraction were mixed with a little cottonseed oil (to facilitate emulsification), emulsified in water with acacia, and fed in four daily doses of 1 gm. each to sheep 779, from July 11 to July 14, 1923. There was no effect from this feeding.

A similar distillate from lot E 8 fed to sheep 782 from January 2 to January 5, 1924, in four doses of 1.25 gm. each (emulsified) produced no effect on the animal.

The remaining vacuum distillates and the residue obtained in the first distillation were fed separately in large doses to this sheep, but in no case was the animal affected. It seemed likely, therefore, that the toxic substance was altered by the distillation.

A small quantity of the undistilled petroleum-ether-soluble material, calculated to be equivalent to 31.34 pounds of green plant, but probably equivalent to much less on account of various losses incidental to chemical processing, was emulsified and fed to sheep 779 in two doses on September 12 and 13, 1923. This treatment appeared markedly to depress the animal, but no trembling was observed, and the feedings could not be continued on account of lack of material.

#### TREMETOL

Fractions E 5. AAB, E 7. 3A, and E 10. AAB, which consisted in part of tremetol and fractions E 7.4A and 7.5A, which were impure tremetol, were fed to sheep 625, 650, 707, 5, 708, and 779. All the sheep were affected and the first five developed characteristic symptoms of trembles; sheep 625 recovered and the remainder died. Sheep 709, a suckling lamb that received the milk of sheep 708 while the latter was sick with trembles, also developed characteristic symptoms of the poisoning.

Many feeding tests have been made on guinea pigs, but these animals have not reacted in any uniform fashion to the doses given. Highly purified tremetol, from a lot used for chemical analyses and

molecular-weight determinations, was injected intraperitoneally into 4 guinea pigs July 8, 1926. Pigs 293, 294, 295, and 296 received respectively 0.2 c. c., 0.1 c. c., 0.15 c. c., and 0.3 c. c. All were made sick, pigs 294 and 295 died in 7 days, and the post-mortem examination,<sup>4</sup> disclosed the nutmeg liver commonly found in trembles cases. The remaining animals that received the larger doses survived. On July 27 these were again injected intraperitoneally, pig 293 receiving 0.25 c. c. and pig 296 receiving 0.3 c. c. Both were made sick. Pig 296 showed very severe symptoms, lost considerable weight, and died August 29.

The autopsy revealed emaciation, anemia, and some enlargement of the liver. The urine was acid and contained albumin, but no acetone bodies. At first, pig No. 293 did not appear greatly affected. He became depressed and finally lost weight and was found dead September 15, seven weeks after the last injection. Autopsy showed emaciation, and anemia. The liver was pale and contained several large, yellowish areas, the kidneys were pale, and the spleen was enlarged and dark. There were three small healed ulcers on the mucosa of the ileum. No odor of acetone was present.

The experimental evidence indicates that tremetol is the substance that causes the disease known as trembles in cattle, horses, and sheep. On account of the small quantity of pure tremetol available it was impossible to conduct confirmatory experiments on large animals during this investigation. Final proof of the causal relationship of tremetol to trembles, therefore, must be delayed until such experiments can be carried out. Efforts are in progress to prepare a quantity of pure tremetol for this purpose.

#### FEEDING OF EXTRACTS THAT DID NOT CONTAIN TREMETOL

All other constituents of the plant were fed at one time or another to sheep, guinea pigs, rabbits, and cats. The only other constituents of the plant that produced death or symptoms of poisoning were the resin acids and the volatile oil. Several feedings of the resin acids to sheep 6, 550, 622, and 679 produced no effect except that in the case of sheep 622 a small but definite depression of the pulse rate was observed. Certainly the resin acids are incapable of producing trembles and probably play no rôle in milk sickness under usual conditions.

The resin acids are, however, toxic to rabbits and guinea pigs, when derived from either green or dried plants.

It is very likely that many of the cases produced in guinea pigs and rabbits in experimental feeding with *Eupatorium urticaefolium*, green and dried, and with extracts made from it, have been the result of poisoning by the resin acids rather than by tremetol. This would account for the toxicity of the dried plant and its extracts to laboratory animals as reported by several investigators, a fact which seemed contrary to the general experience that dried plant would not produce trembles in cattle and sheep when fed in moderately large doses. Drying the plant apparently does not greatly diminish the toxicity of these resin acids.

None of the remaining constituents of *Eupatorium urticaefolium* was observed to be harmful to the animals experimented with even in the very large doses sometimes fed.

<sup>4</sup> Conducted by W. S. Goehenour, to whom the author wishes to express his appreciation.

## GLUCOSIDES

Aqueous extracts that contain the glucosidal substance present in richweed fed to several guinea pigs and to sheep 539, 596, 650, and 6 produced no effect on these animals. This fact considered in conjunction with other evidence as to the character of the toxic principle seems to indicate that this glucoside is not toxic and bears no relationship to trembles.

## ALKALOIDS

The basic substances extracted from lot E 2 were fed to sheep 534. The bases were also present in the aqueous extracts fed as described in the preceding paragraph. No evidence of toxicity was obtained as a result of these feedings, and it is apparent that the basic constituents of richweed are not responsible for the toxicity of the plant.

## ALUMINUM PHOSPHATE

No feedings with aluminum phosphate were made. The extensive feeding experiments reported by Wolf, Curtis, and Kaupp (22), the abandonment of the aluminum hypothesis by Moseley (13), who originated it, the absence of anything in the pharmacology of aluminium compounds that supports the hypothesis, and the discovery of an aluminium-free substance that does produce trembles, all obviate the necessity of experimental work with this compound.

## DRIED AND GREEN PLANTS

Lots E 2 and E 8 were specimens of dried *Eupatorium urticaefolium* that had been collected in the neighborhood of Beecher City, Ill., in 1915, and dried and stored, one for 4 and the other for 8 years before being used in this investigation. No cases of trembles were produced by extracts of these specimens. Eight lots of green plant were used, 4 from Virginia and 4 from Illinois. Of the Virginia collection, 1 was extracted and fed only to guinea pigs, 2 others were fed in substance, and the fourth lot was extracted and fractionated and the fractions fed to sheep. None of these feedings produced cases of trembles. Of the 4 lots of green plant obtained from Illinois, 3 were extracted and produced trembles in sheep; the fourth lot was extracted and fractionated but no cases of trembles were obtained from the feeding of this material. Green plant packed in milk cans and preserved in chloroform water furnished the most active extracts used; other samples of green plant that were mailed to Washington from Illinois as soon as they were collected and which were received within 3 or 4 days and immediately placed in alcohol, were of diminished toxicity.

The color reaction for tremetol has been applied also to specimens of green and dried plants. A sample of the plant collected at the time the material of lot 5 was gathered in Illinois, September, 1920, was shipped in a gunny sack to Washington and was dried in the laboratory. After six years' storage there a portion of it was submitted to the test for tremetol with negative results.

Inasmuch as this plant came from a lot that was very toxic, as demonstrated by feeding tests, the failure to get a positive test for tremetol is quite conclusive evidence that drying destroys this toxic principle. Another sample of dried plant, collected at Woodmont, Va., in June, 1921, and immediately dried in the shade, was tested

after five years' storage in the laboratory. This sample was also negative when tested for tremetol.

Specimens of fresh plant from Arlington County, Va., were tested during the summer of 1926. Several plants collected at different times at one place in Woodmont reacted positively to the tremetol test. A number of other specimens, collected at a point about one-half mile from the first lot, and at different times, gave either a faint or a quite negative reaction to the tremetol test.

#### MILK SICKNESS, TREMBLES, AND ACIDOSIS

Since the first publication on milk sickness by Drake (4) reference has been repeatedly made to a peculiar odor on the breath and eructations of milk-sickness patients and animals with trembles, and to the marked odor observed about corpses and carcasses of victims of the poisoning.

Drake says, "The breath is peculiarly disgusting, even loathsome," and in fact this symptom so uniformly accompanied the malady that it was considered pathognomonic by many physicians.

Walsh (20) suspecting that the foul odor was due to the excretion of acetone by the lungs and recognizing the similarity between certain symptoms of milk sickness and diabetes, diagnosed human milk sickness as acidosis and tested for acetone in the urine. This ketone was found in several specimens.

Jordan and Harris (9), who were in consultation with Walsh, obtained specimens of urine from his cases. In two of these specimens acetone was detected; in a third, taken from a mild case from which the patient had previously recovered, no acetone was detected.

From one of the positive urines R. T. Woodyatt, of the University of Chicago, was able to isolate acetone and identify it by means of the paranitrophenylhydrazine.

Walsh (21) has reported additional cases of milk sickness in which acidosis was observed.

In the course of the present study a great deal of evidence has been obtained which demonstrates conclusively that an acidosis accompanies Eupatorium poisoning. The odor of acetone has been observed on the breath of each sheep that was trembling and in all such cases in which a test was made the presence of acetone in the urine was demonstrated. In a few cases, acetone was tested for and demonstrated in the blood. Inasmuch as symptoms of severe poisoning appear at least 24 hours before acetone may be detected in the urine or on the breath, it seems that the acidosis is a consequence of the poisoning and not one of the casual factors.

The results obtained in the examinations for acetone are presented in Table 5.

TABLE 5.—*Excretion of acetone by sheep poisoned by richweed*

Sheep No.	Date of trembling	Date acetone appeared on breath	Date acetone was found * in urine	Acetone in blood	Termination of sickness
625	Sept. 4, 1921	Sept. 6, 1921	Sept. 6, 1921	Not tested for.....	Recovered.
650	Sept. 13, 1921	Sept. 15, 1921	Sept. 15, 1921	Present.....	Killed for autopsy, Sept. 19, 1921
709	Apr. 21, 1923	Not noted.	Apr. 26, 1923	Not tested for.....	Killed for autopsy.
5	Apr. 16, 1926	Apr. 17, 1926	Apr. 17, 1926	Present.....	Died Apr. 22, 1926.

The effect on the blood-sugar concentration is shown in the case of sheep 5 whose normal blood-sugar content was 0.104 gm. per 100 c. c. On the day when the first trembling was noted the blood sugar had risen to 0.168 gm. This was followed by a fall to 0.138 gm. six days later, due probably to the fact that feeding with the *Eupatorium* extract was discontinued as soon as symptoms of trembling appeared and remedial measures were begun.

### CONCLUSIONS

The chemical and pharmacological data indicate that the constituent of *Eupatorium urticaefolium* which is responsible for trembles is tremetol,  $C_{16}H_{22}O_3$ . Tremetol has the properties of an unsaturated alcohol and is soluble in fats and fat solvents. It may be secreted in milk and would be found in butter made from such milk.

When tremetol is dissolved in petroleum ether and the solution is floated on the surface of sulphuric acid, a characteristic red color is produced.

A volatile oil and a resin acid are also present in this plant. Neither of these is capable of producing trembles in sheep. The resin acid is toxic to rabbits and guinea pigs. Small doses of the volatile oil appear harmless, but larger doses are dangerous.

Other constituents of the plant include fatty acids, especially acetic acid; a crystalline sterol,  $C_{18}H_{30}O$ , melting at  $148-9^{\circ}$  C.; inulin; levulose; an organic base; and a nontoxic glucoside.

When richweed is dried the tremetol content rapidly diminishes and when completely dry the plant is incapable of producing trembles. The dried plant, however, still contains the toxic resin acid and the volatile oil.

Sheep poisoned by richweed or by tremetol develop an acidosis and excrete acetone through lungs and kidneys. The sugar content of the blood is markedly increased. Guinea pigs that were poisoned by the dry plant, with the resin acid, or tremetol, were not observed to excrete acetone.

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## A CYTOLOGICAL STUDY OF CERATOSTOMELLA ADIPOSUM. (BUTL.) COMB. NOV., THE BLACK-ROT FUNGUS OF SUGAR CANE<sup>1</sup>

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91 JAN 1928

### INTRODUCTION

The fungus which is the subject of this study was first described by Butler<sup>3</sup> as *Sphaeronema adiposum*. It is common in the sugar-cane fields of India, and does considerable damage to seed pieces under certain conditions, which Butler fully describes in his article. Normally, however, it is a weak parasite of little or no economic importance.

Perhaps on account of the unusual behavior of the asci and owing to their small size, the true nature of the fungus was overlooked and consequently was not given its proper taxonomic position. Thus, besides establishing the true nature of the fungus, the phenomena observed are of interest with reference to the development of the Ascomycetes in general, and particularly of the Sphaeriales, which have been studied but little cytologically.

The literature dealing with the nuclear phenomena of the Ascomycetes is extensive, covering many different types of various degrees of sexuality. This literature has been thoroughly reviewed by Gwynne-Vaughan.<sup>4</sup> In this paper it will be mentioned only such observations of others as bear directly on those in this study. The cytological work here reported was undertaken as essential in confirming the decision that the so-called pycnidial stage of the "seed-cane" black-rot fungus was in reality a perfect or sexual stage.

### THE HABIT OF THE FUNGUS

The fungus was found in Terrebonne Parish, La., during the winter of 1924, when it caused slight damage to seed cane stored in sawdust. This damage was confined mostly to the cut ends. In the spring of 1926, however, the fungus caused great loss in a field of cane at Ellendale plantation near Houma, La.

Two varieties of sugar cane, P. O. J. 213 and P. O. J. 36, were received from Cairo, Ga., and planted at Ellendale plantation dur-

<sup>1</sup> Received for publication Apr. 18, 1927; issued November, 1927.

<sup>2</sup> The writer is under obligation to Mana K. Sartoris for making the drawings illustrating this paper.

<sup>3</sup> BUTLER, E. J. FUNGUS DISEASES OF SUGAR CANE IN BENGAL. India Dept. Agr. Mem., Bot. Ser., v. 1, no. 3, 53 p., illus. 1906.

<sup>4</sup> GWYNNE-VAUGHAN, H. C. I. FRASER. FUNGI: ASCOMYCETES, USTILAGINALES, UREDINALES. 232 p., illus. Cambridge [Eng.], 1922.

ing March, 1926. About two weeks after planting, a large proportion of the seed pieces became infected by the black-rot fungus. In cases where the soil was not well packed around the seed pieces, the fungus fruited. The long-beaked perithecia were produced in abundance on the cut ends of the cane (fig. 1, A and B), and at times the entire cane was covered by a cottony mass of black hyphae (fig. 1, G and H), which bore over its entire surface the large spiny endoconidia (fig. 1, C to F). The mycelium did not seem to invade the interior of the host but turned the tissue a dark purple color. In advanced stages the cane was nearly black throughout, showed a soft, watery texture, and emitted an odor similar to that produced by *Thielaviopsis ethacetica*.

Ideal conditions for the development of the fungus were found to be frequent rains, with intermittent periods of warm weather, and loosely packed, lumpy soil, which allowed large air pockets around the seed pieces.

In November, just prior to the harvesting of the cane, the amount of damage was estimated as follows: In the case of the variety P. O. J. 36 the stand was reduced to about 20 per cent of normal, and in the case of the variety P. O. J. 213 to about 30 per cent of normal. The pieces that escaped the attack of the fungus produced normal appearing stools.

## CYTOLOGY

### METHODS OF PROCEDURE

The fungus was obtained in pure culture by sowing single endoconidia on nutrient agar in Petri dishes, and by transferring a group of ascospores from the tip of the beak of the perithecium. (Fig. 1, A.) The ascospores, being embedded in a white, fatlike substance which clings to the tip of the beak in a little translucent globule, were easily transferred to Petri plates. Since the globule was held from 3 to 5 mm. above the surface of the plate, most of the cultures were uncontaminated. The perfect stage was produced by a culture that arose from a single endoconidium. Single ascospore cultures were not made. Butler,<sup>5</sup> however, made single-spore cultures from his *Sphaeronema* and found that a culture from one supposed pycnospore produced all of the stages. Cultures were made in Petri dishes, and the material for this study was taken from time to time until all of the stages were obtained. The material was killed in Flemming's solution, embedded in paraffin, sectioned, and stained in the usual manner. Sections were cut from  $4\mu$  to  $7\mu$  thick and were stained in Haidenhain's haematoxylin.

### ASEXUAL DEVELOPMENT

The asexual stage of the fungus has been adequately described by Butler.<sup>5</sup> The vegetative mycelium is mostly uninucleate. When the mycelium is growing rapidly, however, cells may be multinucleate, a condition arising in many cases through fragmentation. The endoconidia, which are binucleate, are extremely variable in size and shape, ranging from elongated hyaline spores resembling the micro-

<sup>5</sup> BUTLER, E. J. Op. cit.

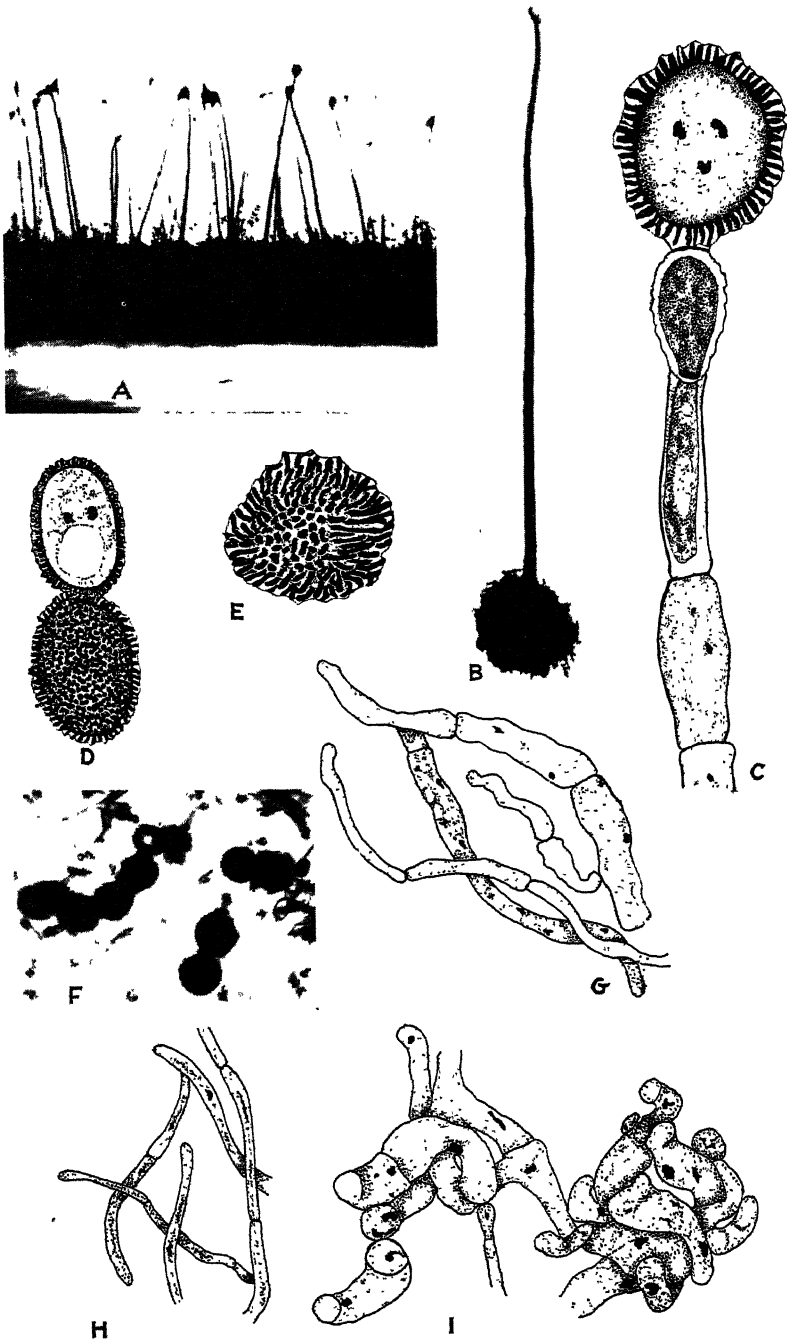


FIG. 1.—A, Habit of black-rot fungus, showing the long beaks with globules of spores at the top.  $\times 4.5$ ; B, a mature perithecium,  $\times 21$ ; C, conidiophore with a mature endoconidium and two others in early stages of formation,  $\times 750$ ; D, the oblong hyaline endoconidia,  $\times 750$ ; E, surface drawing of a mature globose endoconidium,  $\times 750$ ; F, photomicrograph of chains of mature endoconidia,  $\times 260$ ; G and H, vegetative (feeding) hyphae,  $\times 750$ ; I, coils of hyphae which are the perithecium initials.  $\times 750$ .

conidia of *Thielaviopsis ethacetica* to large rounded ones of a deep brown color. (Fig. 1, C to F.) The exospores of the mature conidia are roughened or spiny and very thick. The same conidiophores produce the two kinds of conidia. The hyaline ones are formed first, and later the large, round, deep-brown ones. These endoconidia are formed endogenously (fig. 1, C), and measure  $4.5\mu$  to  $18\mu$  by  $9\mu$  to  $25\mu$ .

#### SEXUAL DEVELOPMENT

##### DEVELOPMENT OF THE PERITHECIUM

The perithecium is initiated by a coil of hyphae (fig. 1, I), in which there appears to be no fusion of nuclei of adjacent cells or of adjacent hyphae, although the coil is formed by somewhat enlarged hyphae from different sources, i. e., not by a single hypha. Coils similar to those described by Elliott<sup>6</sup> for *Ceratostomella fimbriata* were seen, but there was no differentiation of hyphae that could be designated as antheridia and oogonia. The coil becomes rather large and is composed of several layers before the fertile hyphae, which produce the asci, are differentiated. When the coil or perithecium is about 80 microns in cross section, a hypha in the center of the coil stains a deep blue with Haidenhain's haematoxylin. (Fig. 2, A.) This marks the beginning of the ascogenous hyphae. Also at the same time or shortly thereafter a meristematic group of hyphae, which are instrumental in the development of the beak, arise just above the fertile hyphae. (Fig. 2, D.) This area is always directly above the point of attachment of the perithecium to the substratum.

At this time the perithecium is spherical and is covered with radiating long dark-brown hyphae, which give it a *Chaetomium*-like appearance. As it increases in size a cavity is formed at the center (fig. 2, C and D), which is lined by irregular swollen ascogenous hyphae and filled with large thin-walled cells (fig. 2, C to F), which are used up as the fertile hyphae develop. It seems quite evident that these are food-storage cells. In the early stages the ascogenous hyphae are coenocytic. The nuclei are dividing rapidly, in many cases by means of fragmentation. They seem to be in pairs, but this condition may be due to rapid growth. (Fig. 2, C.)

The branching ascogenous hyphae grow across the cavity, between the large thin-walled sterile cells and around the inner wall of the cavity. Whenever the fertile hyphae come in contact with the large sterile cells or with the thick-walled cells of the true wall of the perithecium, they become permanently attached and are more or less independent of the original base. When the point of development at which the asci are being formed is reached, the ascogenous hyphae appear to be rising from all parts of the inner wall of the perithecium. (Fig. 3, A, B, and C.) Asci near the base of the beak are matured first. (Fig. 3, A.) The development continues downward and outward until the sides of the perithecium are reached and the ascogenous hyphae and nutrient cells are exhausted. (Fig. 3, C.)

<sup>6</sup> ELLIOTT, J. A. A CYTOLOGICAL STUDY OF *CERATOSTOMELLA FIMBRIATA* (B. & H.) ELLIOTT. *Phytopathology* 15: 417-422, illus. 1925.

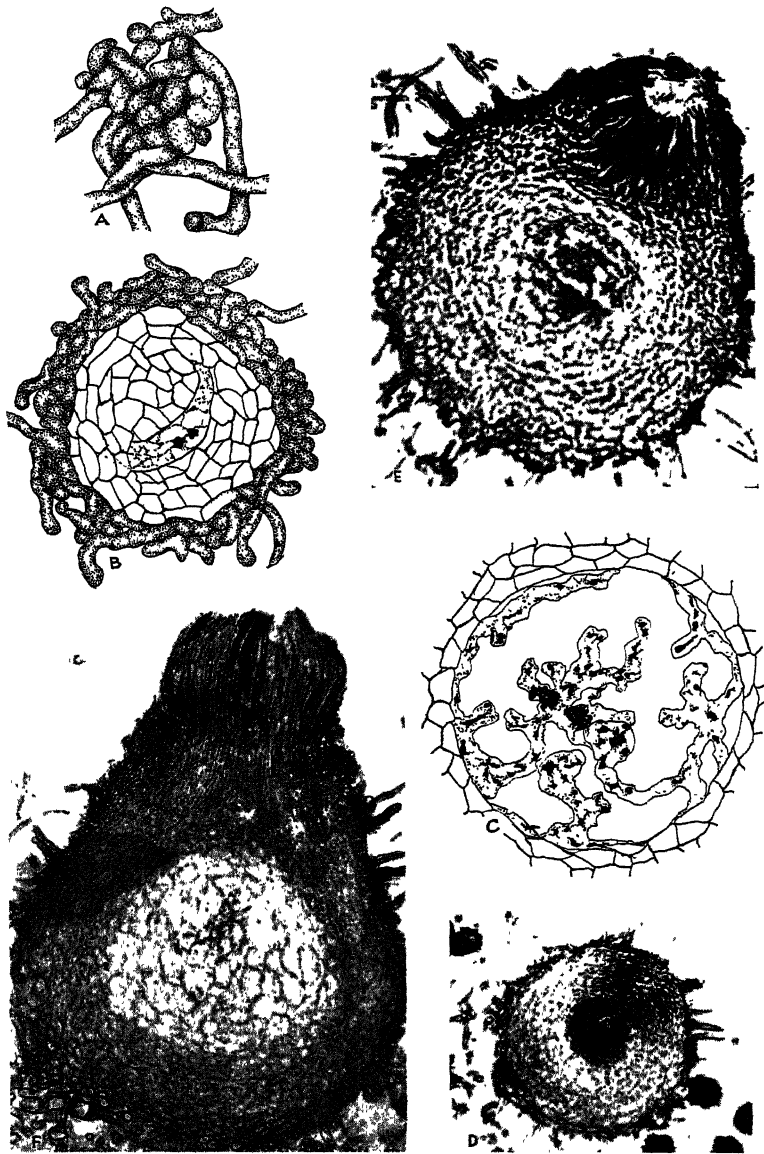


FIG. 2.—A, A coil of hyphae in a more advanced stage than the coils shown in Figure 1, I,  $\times 590$ ; B, a young perithecium, showing the differentiation of the first ascogenous hyphae,  $\times 590$ ; C, a part of the cavity of a young perithecium with branching coenocytic ascogenous hyphae and the thin-walled cells of the cavity,  $\times 590$ ; D, a young perithecium, showing the ascogenous hyphae in the central cavity and above them the meristematic group of hyphae which develop into the beak,  $\times 177$ ; E and F, young perithecia, showing early stages in the development of the beak,  $\times 177$ .

## DEVELOPMENT OF THE ASCUS

The ascogenous hyphae are coenocytic and remain so until the ascus mother cells are about to be developed. Then cross walls are laid down, beginning at the terminal end of each ascogenous hypha, forming binucleate ascus mother cells. (Fig. 3, D and E.) During the growth of the ascus mother cell the two nuclei fuse to form a single fusion nucleus. (Fig. 3, E and F.) This union is followed almost immediately by a series of three divisions, producing eight nuclei. (Fig. 3, G, H, and I.) Then in the usual fashion cell walls are laid down around each nucleus (fig. 3, J and K), cutting out eight irregular spores which later become spherical and then crescent shaped (fig. 3, L). At this time the spores are biseriata and the ascus is cylindrical to pyriform and measures  $10\mu$  by  $20\mu$  to  $12\mu$  by  $25\mu$ . Either while the spores are in a spherical form or later the ascus wall begins to degenerate. In the meantime a translucent vacuolate fatty substance is formed within the ascus which persists even after the wall degenerates, holding the ascospores in groups. (Fig. 3, C.) The ascospores may continue their development for some time after the dissolution of the ascus wall. When mature they measure  $3.5\mu$  by  $6.5\mu$  to  $4\mu$  by  $8\mu$  and are crescent shaped. (Fig. 3, C and M.)

## DEVELOPMENT OF THE BEAK

The development of the beak in the Sphaeriales has not been studied. Elliott<sup>1</sup> studied the life history of *Ceratostomella fimbriata* but did not describe the formation of the beak. As mentioned above, simultaneously with the differentiation of the ascogenous hyphae a meristematic area is formed at the apex of the perithecium. (Fig. 2, D.) The hyphae that form this tissue arise from opposite points of the inner wall of the perithecium. They first grow toward each other, until there is left only a small passage between them. They then turn upward, the hyphae running parallel to each other. (Fig. 2, E and F.) At the same time thick-walled hyphae from the true wall of the perithecium grow parallel to the inner thin-walled hyphae, enveloping them and extending beyond them. (Fig. 3, A, and fig. 4, A and B.) The whole forms a compact, fasciculated, rigid structure. As the beak grows the central canal begins to develop at the base. The central thin-walled hyphae are dissolved (fig. 4, C), so that longitudinal sections and cross sections show a central canal surrounded by layers of thick-walled hyphae (fig. 4, D, and F). When the beak has attained its full height of 2 to 6 mm., that portion of the outer wall extending beyond the growing points spreads out in the form of a fringe. The beak is always developed at right angles to the substratum, since it is not influenced by gravity or light.

Meanwhile the mature spores and the fatty vacuolated substance in which they are embedded completely fill the cavity of the perithecium. This develops sufficient hydrostatic pressure to force some ascospores in a portion of the fatty medium up the canal to the mouth of the beak. Here they gather in a translucent droplet, held in place by the fimbriate appendages. (Fig. 4, E.) The re-

<sup>1</sup> ELLIOTT, J. A. Op. cit.

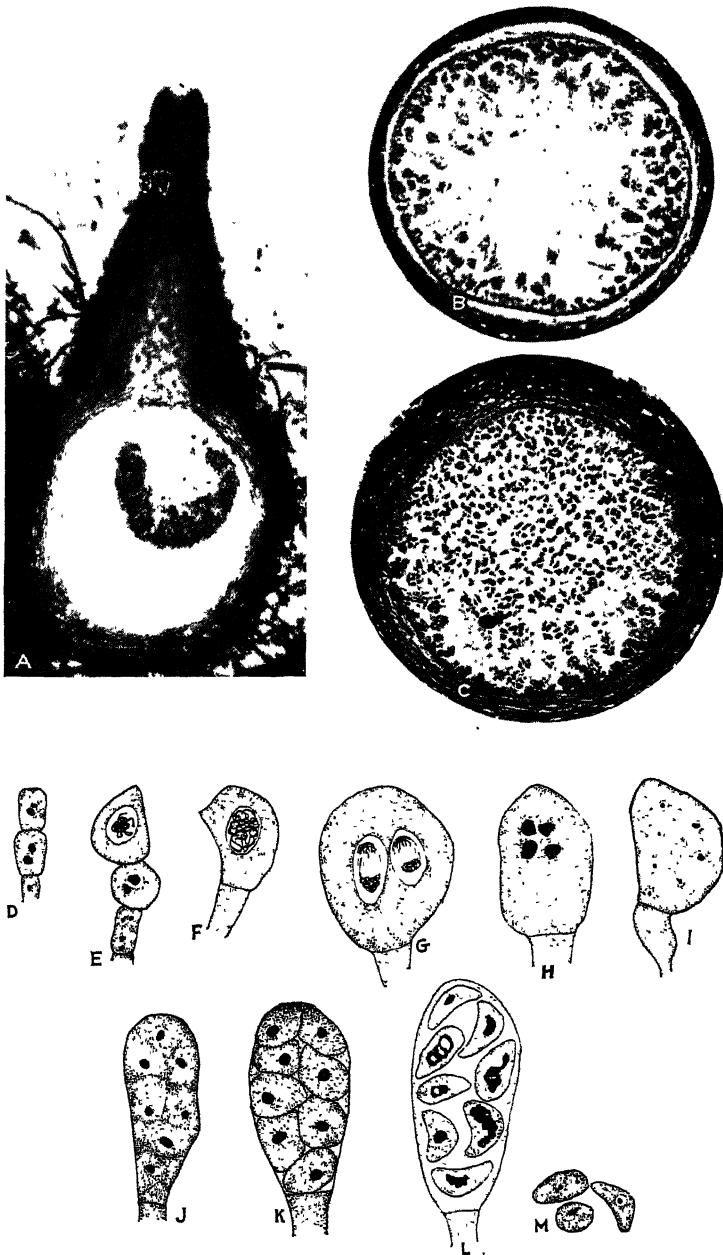


FIG. 3.—A, A later stage in the development of the beak, which shows also the ascogenous hyphae,  $\times 165$ ; B, cross section of a perithecium, showing stages in the maturation of the asci,  $\times 165$ ; C, cross section of a mature perithecium, showing the cavity filled with ascospores and the fatty medium,  $\times 165$ ; D, the binucleate ascus mother cell formed at the end of an ascogenous hypha,  $\times 1100$ ; E, stages of nuclear fusion,  $\times 1100$ ; F, the fusion nucleus,  $\times 1100$ ; G, the first division of the nucleus,  $\times 1100$ ; H, the second division of the nucleus,  $\times 1100$ ; I, the third division of the nucleus,  $\times 1100$ ; J, cleavages in the cytoplasm around the nuclei,  $\times 1100$ ; K, an ascus with eight rounded ascospores,  $\times 1100$ ; L, a mature ascus with eight crescent-shaped spores,  $\times 1100$ ; M, mature crescent-shaped ascospores,  $\times 1100$ .

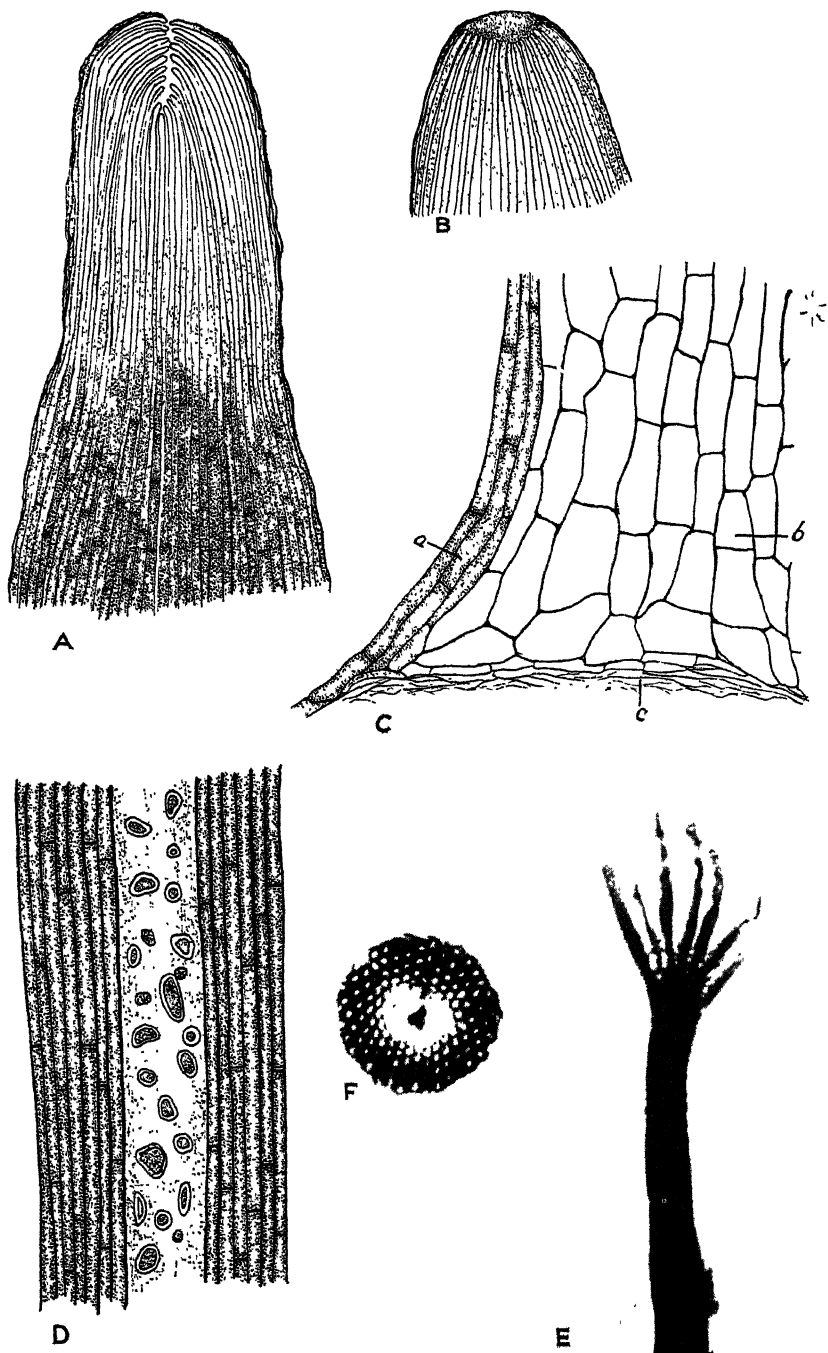


FIG. 4.—A and B, The tip of the beak, showing the growing point enveloped by the hyphae of the true wall of the perithecium,  $\times 770$ . C, A longitudinal section of the beak showing (a) thick-walled cells forming the true wall of the perithecium; (b) large thin-walled cells that are dissolved away, thus forming the central canal; and (c) degenerating hyphae at the base of the beak and lining the cavity of the perithecium,  $\times 1150$ . D, A longitudinal section of a mature beak, showing the thick wall and the central canal with ascospores being forced out,  $\times 770$ . E, The appendages around the mouth of the beak,  $\times 150$ . F, A cross section of the beak, showing the structure of the wall, the central canal, and spores in the canal.

mainder of the ascospores are not disseminated until the perithecium degenerates.

When the droplet is first formed it is white and translucent, but with age it turns yellow and finally brown. This change in color is probably due to the action of oxidases. If the droplet is not disturbed, the original shape is retained with very little loss in size for a long time. The ascospores remain viable for about three months. The fatty substance seems to aid in accomplishing three results—first, to carry the spores to the mouth of the beak; second, to aid in the dissemination of the spores by sticking to insects that may crawl over the culture; and third, to keep the spores from drying out.

#### SUMMARY

The results of this study show that the fungus described by Butler as *Sphaeronema adiposum* is in reality an ascomycete belonging to the genus *Ceratostomella*. Its name therefore becomes *Ceratostomella adiposum* (Butler) Sartoris, comb. nov.

Under certain conditions the fungus causes great loss to seed cane, but normally it is a weak parasite.

The perithecium is initiated by a coil of enlarged hyphae, but differentiation of the ascogenous hyphae does not take place until the young perithecium is quite large. The ascogenous hyphae are coenocytic during most of their development, but just prior to ascus formation cross walls are laid down, forming binucleate ascus mother cells. These nuclei fuse to produce a fusion nucleus, which by three divisions forms eight nuclei which become ascospore nuclei. The beak is formed by a meristematic group of hyphae which are differentiated at the apex of the young perithecium. The ascospores are forced up the canal of the long beak by the development of hydrostatic pressure in the base of the perithecium.



# SOIL FACTORS INFLUENCING THE DEVELOPMENT OF THE MOSAIC DISEASE IN WINTER WHEAT<sup>1</sup>

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## INTRODUCTION

Early studies by McKinney (8)<sup>3</sup> indicated that environmental factors during the seedling stage are important in the development of the rosetted condition of the mosaic disease of winter wheat. Beginning with a normal seeding date, he found that the percentages of rosetted plants from periodic seedings were less in the late sowings than in the early ones. He found further that the disease practically was controlled where emergence did not occur until the following spring.

The causal agent of the wheat mosaic disease, referred to in previous publications (8, 10), is soil borne, and the disease has its natural origin or point of attack on the underground parts of plants in the seedling stage. In fact, until the recently successful inoculation experiments by McKinney (10) the only method of obtaining the disease was by sowing susceptible varieties in infested soil during the natural growing season for winter wheat. Even now this is the only method whereby relatively high percentages of diseased plants can be obtained. The very important soil relation of this disease certainly possesses especial significance and furnishes an opportunity for study along lines which thus far have been either impossible or difficult for other virus diseases of the mosaic type.

What are the possible rôles which environmental factors are playing in this disease? Are the two distinct varietal responses, namely, the rosette and the mosaic-mottling phases, influenced by the same environmental factors? If so, are they affected in the same way and to the same degree? It was in an attempt to answer these questions that this investigation was started. A study of the environmental conditions with particular reference to the soil phase was essential to a proper understanding of the disease and of its occurrence and distribution in general and in restricted areas. Accordingly, experiments have been conducted on the influences of constant and fluctuating soil temperatures and soil moistures during the critical stages for infection. The influences of the stage of seedling development and of the time factor in relation to soil temperature have been followed. Further, the effects of surface sterilization of the underground parts of infected seedlings with bichloride of mercury have been determined. Although these studies are rather preliminary, it is believed that the results are of sufficient importance to warrant their publication at this time.

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<sup>2</sup> The writer is grateful to H. H. McKinney for assistance in certain phases of the experiments and for suggestions concerning the manuscript; also to C. R. Ball and A. G. Johnson for editorial suggestions.

<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 613

## EXPERIMENTS

The studies reported in this paper were conducted in experimental plots out of doors at Madison, Wis., during the winter-wheat seasons of 1923-24 and 1924-25. All of the experiments reported have been conducted in season; that is, the grain was sown during the fall and the plants passed through their usual period of winter dormancy. Infection occurs in the seedling stage during the autumnal period, and consequently the most attention has been focused on this stage and on this period.

Two very susceptible varieties of winter wheat, namely, Harvest Queen and Currell, have been tested simultaneously in all experiments except one. The Harvest Queen variety is an excellent one for expressing both the rosette and the mosaic-mottling phases, and the Currell variety is an excellent one for expressing only the mosaic mottling. These various symptoms have been described fully in the previous papers (8, 10, 11, 12). All of the available evidence seems to indicate that the rosette and the mosaic-mottling phases are different expressions of the same disease.

The naturally infested soil used in these experiments was transported to Madison from the American Bottoms of the Mississippi River near Granite City, Ill., across the river from St. Louis, Mo. This soil is a fine sedimentary type and is spoken of as a heavy gumbo. The very consistent and uniform results obtained in all the experiments indicate that this soil was more or less uniformly infested. Fertile loam soil obtained in the vicinity of Madison, Wis., served as a control in the experiments.

The air and soil temperatures were accurately recorded by thermographs which were periodically checked. The soil-temperature data represent the conditions at a depth of 2 inches. Daily fluctuating temperatures have been converted by taking a mean of the maximum and minimum daily temperatures and by expressing these in terms of a mean weekly value. Any method of evaluating fluctuating temperatures is open to criticism, but the method employed here was thoroughly adequate for the purpose of the experiments. Only the soil-temperature data for the seedling stage have been included in this paper. The air-temperature data were very similar to the soil-temperature data, with the exception, of course, of greater daily fluctuations.

## TANK-TRANSPLANTING EXPERIMENT, 1924-25

## METHODS

The studies of controlled soil temperature and moisture were combined and conducted jointly. The Wisconsin soil-temperature tanks, as described by Jones (4, 5) and as modified by Dickson (1), McKinney (9), and others, were employed. In order that the seedlings might experience the usual fall conditions out of doors and thereby develop sufficient hardiness to withstand the severity of the winter, these experiments were not conducted in the greenhouse. Four of the tanks were arranged side by side in the outdoor experimental plot, and were securely walled with boards, covered with tar roofing, and further insulated with sawdust between the tanks and the walls. The 30° and 23° C. tanks were electrically heated and thermostatically controlled; the 16° tank was regulated by the

adjustment of the inflow of water from the city water main; and the 10° tank was supplied with cracked ice several times a day, depending upon weather conditions. All regulations were made on the basis of the soil temperature 1½ inches beneath the surface and in the center of the cans. Considering the outdoor conditions, the soil temperatures were surprisingly constant. The tanks intended to be held at 30°, 23°, and 16°, respectively, were regulated to within an average of 0.5° to 1.0° of the desired temperature. Somewhat greater variations occurred in the tank at the lowest temperature, in which the temperature ranged from 8° to 12°, although there was a fairly constant 10° value during most of the period. The extremes of this temperature range occurred during relatively short periods when the ice was applied or when the sun shone intensely. The spreads between the temperatures of the various tanks, however, were sufficiently wide to offset the influence of such minor variations and to enable the experiments to serve their purpose.

The infested soil employed in each series of tank experiments was thoroughly pulverized, screened, mixed, and adjusted to the desired moisture content as expressed in percentage of its moisture-holding capacity. The moisture-holding capacity of this soil was 60 per cent. A weighed quantity of soil was placed in each container, and the soil moisture was kept as nearly constant as possible during the course of the experiments by daily weighing the cultures and bringing them to constant weight with water of the same temperature as the tank. Tap water was used in all the adjustments, and it was applied at the surface. Despite the variations in moisture distributions and the crude method used in attempting to regulate it, the adjusted soils certainly represent low, midheight, and high moisture contents. Granite City soil adjusted to a soil-moisture content of 42 to 45 per cent of its moisture-holding capacity possesses ideal friability for tillage and plant growth; that at 52 to 58 per cent of capacity represents the highest moisture content at which the soil can be satisfactorily worked; whereas soil at 30 to 32 per cent of capacity is extremely dry, and soaked seeds require considerable periods for germination in it.

The fertile loam soil used for checks possessed a moisture-holding capacity of 30 per cent. Checks were employed at only one soil-moisture content, namely, 42 to 45 per cent of the moisture-holding capacity.

Containers of two sizes were employed. In the first experiment, ordinary No. 2 pea cans were used; in the second, metal containers 6 inches in diameter and 10 inches in depth were used. A distinct advantage is offered by the small containers in that they permit of easy removal and manipulation of plants with the least possible injury. On the other hand, the increased number of small containers greatly increases the routine daily weighing and watering, and this is a decided disadvantage. In general, however, the No. 2 pea cans are well adapted to seeding, transplanting, and overwintering studies such as are reported in this paper.

The grain to be sown was hand picked and soaked overnight in water at room temperature. This was done in order to facilitate and hasten germination, especially in the soil adjusted to the lowest moisture content, thus giving the seedlings the longest and most uniform period possible under the controlled conditions. In each

of the smaller containers 12 kernels were evenly distributed, and five such containers constituted a group. In each of the larger containers 60 kernels were similarly sown, and only one container was used for each combination. Thus, a total of 60 kernels was tested for each variety in each moisture content at each temperature. In all cases the grain was sown at a uniform depth of 1.5 inches.

At night, and during the day before showers occurred, the tanks were covered with a waterproof canvas which protected the plants against all moisture other than that which was applied to maintain a constant weight. The canvas was spread over a ridgepole fully 4 feet above the plants, and the sides of the canvas were securely attached to the wall surrounding the tanks. The ends of the canvas were left open to permit normal air circulation.

Sowings in the first experiment were made September 23, 1924, and in the second experiment November 1, 1924. Thirty days from date of seeding the plants were carefully removed, the soil was washed away, the plants were allowed to soak in several changes of tap water, all soil particles were removed by means of agitation in water with a camel's-hair brush, and the plants were further rinsed in running tap water and transplanted to metal containers filled with disease-free soil. The plants showing severe *Helminthosporium* infection on the underground parts were segregated and transplanted separately. Holes were cut in the bottoms of the cans for drainage, and slots were cut in the side from the top to the soil line to permit surface drainage. The cans then were buried in the ground to the soil line, mulched with hay, and allowed to overwinter in this condition.

#### EFFECTS ON ROOT DEVELOPMENT

The two varieties of winter wheat tested proved very sensitive to different soil temperatures, which indicated a different type of metabolism for the seedlings growing under each different set of environmental conditions described. The results obtained are shown in Table 1, and they agree well with those published by Dickson (1), McKinney (9), and others.

In general, the germination of both the Harvest Queen and Currell varieties was hastened by high soil temperature and high soil moisture. Germination was somewhat poor and erratic at both the midheight and high-moisture contents in a temperature of 30° C. and at the low moisture contents in temperatures of 10° and 16° C. No germination whatever occurred at the low moisture contents in temperatures of 23° and 30° C., despite the fact that the seeds were soaked overnight in water at room temperature before sowing. The seedlings emerged promptly and uniformly, except those in the soil of low moisture content at temperatures of 10° and 16° C. Prolonged periods were required for the emergence of these seedlings. At the time of transplanting, only about one-third to two-thirds of the germinated kernels in these cases had produced emerging seedlings.

The type and degree of root development were distinctly different at the high and at the low temperatures. No very marked variations, other than that of extent of growth, occurred with the different moistures at any particular temperature. At the two lower temperatures, 10° and 16° C., the root systems were well developed, very

extensive, long, white, fleshy, smooth, and nonbranching. At the two higher temperatures, 23° and 30° C., the root systems were poorly developed, short, brown, fibrous, rough, and branching. The type of soil appeared to influence the length of root development to some extent, the average root system being 1 to several inches longer in the control sandy-loam soil than in the infested gumbo soil.

TABLE 1.—*Influence of constant soil temperatures and constant soil moistures on the development of the mosaic disease of Harvest Queen and Currell winter wheats in naturally infested soil*

Variety and soil temperature	Soil moisture	Germination	Emergence	Time elapsed from seeding to emergence	Seedlings transplanted Oct. 23, 1924						Mosaic disease in 1925				
					Leaf stage of development	Length of—			Helminthosporium infection	Number of plants surviving	Mottling		Rosette, June 4		
						Toys	Roots	Subcoronal internode			Mild <sup>a</sup>	Severe <sup>b</sup>		April 28	May 12
Harvest Queen.	P.ct	P.ct.	P.ct.	Ds.		Ins.	Ins.	Inches	P.ct	P.ct		P.ct.	P.ct.	P.ct.	
10° C	{ 30	{ 83.3	{ 32.0	{ 30	1st.	4.5	3.5	0	0	0	50	0	0	0	
	{ 42	{ 100	{ 100	{ 11	2d.	4.5	3.5	0	0	0	60	0	<sup>d</sup> 1.7	<sup>e</sup> 1.7	
	{ 52	{ 100	{ 100	{ 9	3d.	5	4.5	0	0	0	60	100	100	<sup>e</sup> 37.7	
16° C	{ 30 <sup>h</sup>	{ 86.7	{ 66.7	{ 19	1st.	4.3	3.5	.1	15.0	0	40	0	0	0	
	{ 42	{ 100	{ 100	{ 7	3d.	6.5	4	.0.25 to 1	15	13.3	60	0	13	13	
	{ 52	{ 98.3	{ 100	{ 5	3d.	7	5	.25 to 1	13.6	3.4	59	100	100	<sup>k</sup> 58.5	
23° C	{ 30	{ 0	{ 0	{ 0	2d to 3d.	8	3.5	.25 to 1	32.7	16.4	55	0	0	0	
	{ 42	{ 91.6	{ 100	{ 3	4th.	9	5.5	.1 to 1.5	0	56.7	60	0	0	0	
	{ 52	{ 100	{ 100	{ 3	4th.	9	5.5	.1 to 1.5	0	56.7	60	0	0	0	
30° C	{ 30	{ 0	{ 0	{ 0	1st to 3d.	7.5	3	.1	31.3	3.1	32	0	0	0	
	{ 42	{ 53.3	{ 100	{ 7	4th.	9	5	1 to 1.5	18.4	36.7	49	0	0	0	
	{ 52	{ 81.7	{ 100	{ 4	4th.	9	5	1 to 1.5	18.4	36.7	49	0	0	0	
Currell:															
	{ 30	{ 70	{ 45.2	<sup>e</sup> 30	1st to 2d.	---	3-4	---	0	0	42	0	0	0	
	{ 42	{ 98.3	{ 98.3	11	3d.	---	4	---	0	0	60	0	<sup>d</sup> 5	---	
10° C	{ 52	{ 98.3	{ 98.3	10	3d.	5.8	4	0	0	0	59	0	50	---	
	{ 30	{ 46.7	{ 46.7	<sup>e</sup> 19	1st to 2d.	4.5	3	0	14.3	0	28	0	0	---	
	{ 42	{ 98.3	{ 98.3	7	3d.	6.5	4	.25 to .75	10.2	0	59	17.7	16.3	---	
16° C	{ 52	{ 85	{ 85	5	3d.	7	5	.25 to .75	17.6	0	51	18.5	50	---	
	{ 30	{ 0	{ 0	{ 0	3d to 4th.	8.5	3.5	.25 to 1	0	51.3	39	0	0	---	
	{ 42	{ 65	{ 65	7	4th.	9	4	.50 to 1	0	73.3	55	0	0	---	
23° C	{ 52	{ 91.6	{ 91.6	3	4th.	9	4	.50 to 1	0	73.3	55	0	0	---	
	{ 30	{ 0	{ 0	{ 0	2d to 3d.	8.3	3	.125 to 1	0	13.3	15	0	0	---	
	{ 42	{ 25	{ 25	8	4th.	9.5	5	.1 to 1.5	0	68	25	0	0	---	
30° C	{ 52	{ 41.7	{ 41.7	4	4th.	9.5	5	.1 to 1.5	0	68	25	0	0	---	

<sup>a</sup> One to several faint lesions on coleoptile, outer sheaths, subcrown, and roots.

<sup>b</sup> Coleoptile dark brown or black and numerous lesions on main tissue, outer sheaths, subcrown or roots.

<sup>c</sup> Very irregular emergence.

<sup>d</sup> Faint mottling.

<sup>e</sup> Intermediate type.

<sup>f</sup> Conspicuous mottling.

<sup>g</sup> Typical rosette, 30.2 per cent; intermediate, 7.5 per cent.

<sup>h</sup> Twelve recently germinated and weakly plants not transplanted.

<sup>i</sup> General.

<sup>j</sup> Variable.

<sup>k</sup> Typical rosette, 47.2 per cent; intermediate, 11.3 per cent.

<sup>l</sup> Midcolor mottling.

The subcoronal internode responded readily to the different environmental conditions. On the whole, the structures were more elongated in the Harvest Queen variety than in the Currell. With the high soil-moisture content, at the two high temperatures, 23°

and 30° C., the elongation was greatest, practically all the plants showing such internodes measuring 1 to 1.5 inches in length. With the median soil-moisture content at the same two temperatures, on the other hand, these structures generally were shorter and considerably more variable in length. Elongation was reduced still further and appeared more variable at 16° C., whereas at 10° C. it was practically inhibited. Soil temperature and soil moisture, therefore, are important factors influencing the development of the subcoronal internode, but the large variations frequently occurring in seedlings at a particular temperature-moisture combination indicate that other factors also operate.

The development of the coleoptile is just the opposite of that of the subcoronal internode. High temperatures of 23° to 30° C. inhibited rapid elongation of the coleoptile, the growing point of the culm generally rupturing the coleoptile before emerging from the soil. At 16° and 10° C., on the other hand, the elongation of the coleoptile proceeded at a much faster rate than that of the culm, and the subsequent rupture of the coleoptile did not occur until some time after its emergence from the soil.

The largest production of tops, expressed in terms of average length from the first node to the tip of the longest leaves, occurred at 23° and 30° C., followed in turn by that at 16° and 10° C. Furthermore, the length varied more or less directly with the percentage of moisture present in the soil. No tillers had developed in either variety at the time the plants were removed from the tanks, namely, 30 days after date of seeding.

The Harvest Queen variety withstood the severity of the winter at Madison, Wis., much better than the Currell variety. In the second tank series, when the seedlings experienced relatively low air temperatures during several nights prior to their removal, interesting correlations were noted between low-temperature injury and the conditions under which the seedlings were growing. Previous high soil temperature and high soil moisture predisposed the seedlings to severe winter injury. For example, at 30° C. the Currell seedlings in soil with the high and median moisture contents were killed, whereas those in soil of low moisture content showed slight top injury, but a weakening of the base at the soil line. Similar though less severe relations were noted at 23° C. At 16° C. the seedlings in the soil of high moisture content were generally injured and weakened at the bases, but no injury occurred at the other two soil-moisture contents. No injury whatever was noted in any of the seedlings grown at a temperature of 10° C.

In general, stronger, healthier, stockier, more vigorous, and darker green plants were obtained at the two low temperatures, 10° and 16° C., than at the two higher temperatures, 23° and 30° C. The plants grown at the higher temperatures were rather spindling, somewhat lighter green in color, and possessed a poorly developed root system as compared to the others. In addition, they were quite generally and heavily infected with *Helminthosporium*.

#### EFFECTS ON DISEASE DEVELOPMENT

Results obtained from the Harvest Queen variety of wheat are shown in Table 1 and in Figures 1 and 2. It is very evident that the disease developed only in plants which passed their early seedling

stage within restricted soil-temperature and soil-moisture ranges. At the two high soil temperatures, 23° and 30° C., neither rosette nor mottling nor any indications of them appeared, whereas at the two low soil temperatures, 16° and 10° C., rosette and mottling occurred. A high soil-moisture content favored the disease, whereas its occurrence was less marked at a medium soil-moisture content. No indications of either rosette or mottling occurred at the low soil-moisture content, namely, 30 per cent. The soil-moisture relations appeared very definite and important. The percentage of plants manifesting the rosetted condition is higher and the type of rosette is more extreme at 16° than at 10° C.

Comparing the results in the high soil-moisture content at the two favorable soil temperatures, it will be seen that 37.7 per cent of the plants grown at 10° C., and 58.5 per cent of those grown at 16° C., showed the rosetted condition. In both cases approximately three-

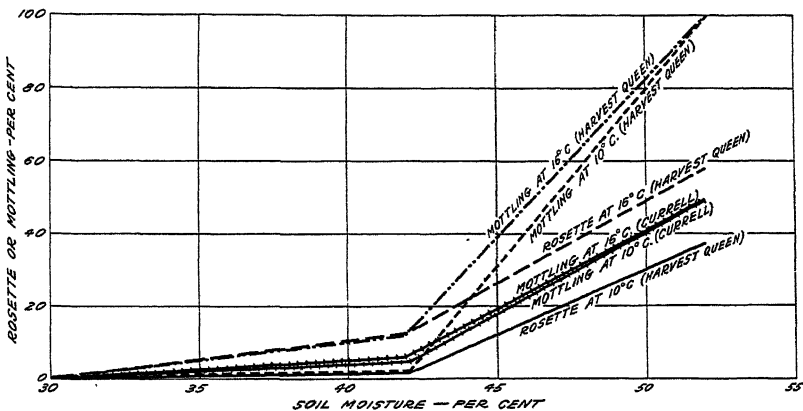


FIG. 1.—Influence of constant soil temperatures and constant soil moistures on the development of the mosaic disease of winter wheat, as expressed in terms of rosette and mosaic mottling by the Harvest Queen variety, and of mosaic mottling by the Currell variety, when grown in naturally infested soil for a period of 30 days from date of seeding, 1924. (Same data plotted differently in fig. 2)

fourths of the plants represented in these percentages manifested distinct rosette symptoms, the remaining one-fourth representing somewhat less extreme types. Under medium soil-moisture content, at each of the two favorable low temperatures, there was a marked reduction in rosetted plants, the percentage having decreased to 1.7 and 13 per cent, respectively.

Up to this point only the rosette phase of the disease has been considered. It is evident from a further examination of the data in Table 1 that the soil-temperature range for mosaic mottling is the same as that for the rosette condition. Whereas the percentages of rosetted plants in the soil of highest moisture content were higher at a temperature of 16° than at 10° C., the percentages of mosaic-mottled plants under similar conditions were the same at both temperatures, 100 per cent. Thus, the mottling phase is more general than the rosetted phase. Furthermore, the mottling in both instances was abundant at different observations extending over a period of several weeks. With a medium soil-moisture content, at the two favorable low soil temperatures, the percentages of mottled plants were greatly reduced, only the rosetted plants showing mot-

ting. The soil-moisture relations for mottling, therefore, resemble those for rosette.

Helminthosporium infection was severe in the infested soil under both the medium and high moisture contents at temperatures of 23° and 30° C. Under these conditions the coleoptiles were entirely brown or black and the subterranean parts showed numerous severe lesions. No indications of the mosaic disease either in the form of rosette or of mottling appeared on such plants. Furthermore, the plants at the soil temperature of 16° C. which showed comparatively mild Helminthosporium infection had neither more nor better rosette or mottling symptoms than those which were free from Helminthosporium. Comparing the soil-temperature range and the soil-

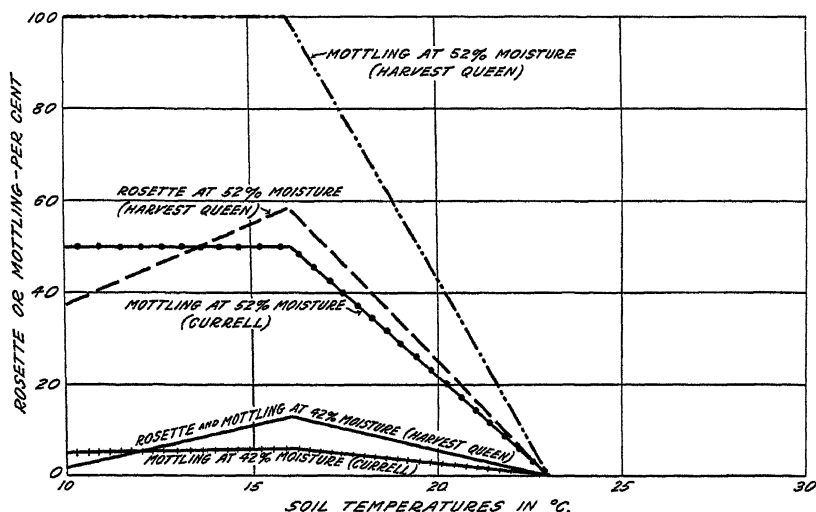


FIG. 2.—Influence of constant soil temperatures and constant soil moistures on the development of the mosaic disease of winter wheat, as expressed in terms of rosette and mosaic mottling by the Harvest Queen variety, and of mosaic mottling by the Currell variety, when grown in naturally infested soil for a period of 30 days from date of seeding. (Same data plotted differently in fig. 1)

temperature optima for the Helminthosporium disease of wheat, as obtained by McKinney (9), with those for the mosaic disease, as obtained in these present experiments, it is evident that the temperature relations of the two diseases are very different. This is further conclusive evidence that Helminthosporium is not the cause of the mosaic disease, as McKinney, Webb, and Dungan (12) previously have shown that it is not the cause of rosette.

The results with the Currell variety are assembled in Table 1 and the curves plotted to illustrate these results appear in Figures 1 and 2. Soil temperatures and soil moistures which were favorable to both the rosette and mottling phases of mosaic disease in the Harvest Queen variety also are favorable to the mottling of the Currell variety. No mottling occurred at 23° and 30° C. The curves representing mottling in Currell in the corresponding soil moistures, at temperatures of 10° and 16° C., are practically identical and are very similar to the curves for rosette at a temperature of 16° C. The number of mottled plants in each case is 6 to 8 per cent less than the number of rosetted plants. Unlike the rosette phase in Harvest Queen, which

occurred in higher percentages at 16° than at 10° C., the mottling in Currell occurred in approximately equal quantities at the two soil temperatures. The two different soil temperatures, however, did influence the intensity of the mottling, even if they did not appreciably alter the percentages. For example, in the medium and high soil-moisture contents, respectively, the mottling was faint to mid-mottley at a temperature of 10°, as compared with midmottley to conspicuous at a temperature of 16° C.

Soil moisture proved to be equally as important a factor in the mottling of the Currell variety as in the rosette and mottling of the Harvest Queen variety. At the favorable temperatures no mottling whatever was evidenced with the low soil-moisture content, despite the fact that the plants were well into the first-leaf stage of development at the time of removal from the infested soil. Only 5 to 6 per cent of the plants showed a faint to midcolor mottling at the medium soil-moisture content, whereas approximately 50 per cent showed a midcolor to conspicuous mottling at the high soil-moisture content. The effects of the soil-moisture relations were very striking.

The Harvest Queen and Currell plants developed normally in the check soil, and the plants at each soil temperature were very similar to the corresponding plants in the infested soil adjusted to medium or high moisture contents. In no case was there the slightest indication of rosette or mottling of the plants which served as controls.

#### FIELD EXPERIMENT ON DATE OF SEEDING, 1924-25

Eight successive seedings of Harvest Queen and Currell winter wheats were made in infested soil, at two-week intervals during the fall of 1924, beginning August 4. The sowings were made in flats measuring 12 by 14 by 8 inches, and the infested soil composed the upper 4-inch layer in the flat. Disease-free Madison loam made up the lower 3-inch layer in the flat. This method consistently has given results similar to those obtained where the infested soil filled the entire depth of the flat. Fifty hand-picked kernels of the particular variety were sown in each flat, 25 kernels in each of two rows, at a depth of 1.5 inches. The soil was watered frequently, depending upon the degree of dryness. The flats were sunk in the ground at a slight angle and two holes were bored directly above the soil line in the lower end of each flat. This insured elimination of surface water before freezing was possible, and this precaution, and the use of a hay mulch, minimized subsequent winter injury to the plants.

#### EFFECTS ON HOST DEVELOPMENT

The data concerning this experiment are given in Table 2. The percentage of germination and the number of days before emergence were variables. In general, however, the best germination occurred in the early seedings and poorest germination occurred in the late seedings. Similarly, the number of days required for emergence was smallest in the two earliest seedings and somewhat greater in the later seedings.

The Currell plants of the seventh seeding date (October 27) were severely injured by air temperatures below freezing November 7, 8, and 9, and they were killed during the winter. Injury was not as



In the case of Harvest Queen, it is interesting to note that no plants of the August 4 seeding showed the rosetted condition, and only a relatively few, 2.3 per cent, of the August 18 seeding showed it. These field results agree excellently with those obtained in the experiments under controlled soil temperature and moisture. The percentages of plants showing rosette in the next four dates of seeding, September 1, 15, and 29 and October 13, respectively, were uniformly high, ranging from 75 to 82 per cent. In these sowings, the mean weekly soil temperature ranged from 16° C. in early September to 10° C. in the middle of October. The rosette symptoms in plants from the September 15 and 29 seeding dates were very pronounced, and proliferation at the crown was excessive. Prolif-



FIG. 3.—Influence of soil temperatures, under field conditions, on the development of the mosaic disease of winter wheat, as expressed in terms of rosette in the Harvest Queen wheat sown on different dates in naturally infested soil, in 1924

eration in a few plants of the October 13 seeding equaled that in the previously mentioned plants, but, in general, it was less. From the last seeding (October 27) in which the seedlings withstood the winter, the rosetted plants totaled 47.4 per cent, which was a considerably lower value than that furnished by the four earlier sowings. The symptoms also were much less marked.

A very faint, questionable, and not general mottling occurred in the plants from both the August 4 and August 18 sowings. The mottling in the seedlings of September 1, 15, and 29 and October 13 and 27 was midmottley to conspicuous and occurred generally. Thus in the two earliest seedings, in which the young seedlings experienced a mean weekly soil temperature of 21° C. and above, little or no rosette developed, and the mottling was very faint. The relatively low temperature for a few hours each night probably has an active

influence and may account for the faint mottling and the mere trace of rosette. These results agree well with those obtained in the tank experiments in which the soil temperature was held constant.

The Currell variety showed different degrees and percentages of mottling in the several sowings. The mottling was faint to mid-mottley and not general in the two August sowings, midmottley to conspicuous and general in the next two, and conspicuous and general in the last two. These observations were made May 1, and those made May 13 showed that the intensity of the mottling was about equal in each of the different sowings. Thus it seems that mottling in the Currell variety can develop during the seedling stage at slightly higher mean soil temperatures than can either mottling or rosette in the Harvest Queen variety. These relations are slightly at variance with those shown in the constant soil-temperature studies, where the soil-temperature and soil-moisture range was the same for both mottling and rosette. In this connection, however, later results show that infection necessary for mottling may take place at later seedling stages, in shorter time intervals, and over a wider temperature range, than that for rosette. This would tend to explain the apparent discrepancies.

While the results of the different periodic sowings have been considered thus far on the basis of soil temperature, it is realized that the duration of exposure to infection, as related to environmental conditions, is an important one. However, the data from this experiment do not throw any light on this particular angle of the problem. In the transplanting experiments which are described next, the effect of varied duration of exposure to infection at different periods during the fall has been studied and the data obtained are significant.

#### FIELD TRANSPLANTING EXPERIMENTS, 1923-24

On October 3, 1923, Harvest Queen wheat was sown in infested soil and in steam-sterilized Madison soil used as a control. One hundred No. 2 pea cans filled with the infested soil and 200 such cans filled with the control soil were employed. Seven kernels were sown in each container, and periodic watering kept the soil in good condition.

Transplantings were made at regular weekly intervals during the fall and winter, but more frequently during the earliest stages of seedling development. Each transplanting involved 60 seedlings, namely, 20 seedlings from noninfested soil to infested soil, 20 seedlings from infested soil to noninfested soil, and 20 seedlings from noninfested soil to noninfested soil, to serve as controls.

The seedlings were removed and transplanted with the greatest care in all cases. The entire contents of the cans were removed, the mass was washed carefully until the roots of the plants were clean, the roots of each seedling were washed repeatedly, and all particles of soil were removed with a camel's hair brush; and the underground parts of the seedlings were sterilized for five minutes in 1:1,000  $\text{HgCl}_2$  and finally washed repeatedly in running tap water. The seedlings were transplanted to the proper soil in similar containers, five seedlings being placed in each.

Immediately after the original seeding and after the different transplantings, the containers were buried in the ground to their

soil line, and slots were cut from the upper edges of the cans to the soil line to permit drainage of surface water. In all cases the plants were well mulched with a light layer of hay to minimize possible winter injury.

#### EFFECTS ON HOST DEVELOPMENT

In considering the results of this transplanting experiment, it should be kept in mind that all the seedlings were made on a single date and that the age and development of the plants therefore were different at each successive transplanting. The seed was sown, however, on a desirable planting date for winter wheat in southern Wisconsin, and the development of the plants thus approximated that occurring under field conditions. The first four transplantings were made at intervals of three to four days. Beginning October 16 they were made at weekly intervals until April 4, but the results presented include only those to December 4. The early fall period proved to be the important period, and the presentation of only the early data is adequate. The data obtained in this experiment are given in Table 3 and the curves appear in Figure 4.

TABLE 3.—*Influence of the duration of exposure to infection and the stage of seedling development, in relation to daily fluctuating soil temperatures, on the development of the rosette phase of the mosaic disease of Harvest Queen wheat, as determined by transplanting seedlings from or to naturally infested soil at different dates during the fall of 1923, as all seedlings made October 3*

Date of transplanting	Age of plants from date of seeding (days)	Stage of development	Period	Average soil temperatures (° C.)			Percent-age of plants showing rosette when transplanted from—	
				Mean	Maximum	Minimum	Noninfested soil to infested soil	Infested soil to noninfested soil
Oct. 5	2	Kernel swollen but not germinated.....	Oct. 5-7.....	.....	.....	.....	100	0
Oct. 8	5	Coleoptile 3 to 4 mm. long.....	Oct. 8-11.....	14.0	18.3	9.5	95	0
Oct. 12	9	Plumule still enclosed; coleoptile 6 to 13 mm.....	Oct. 12-15.....	10.8	14.3	7.2	90	0
Oct. 16	13	1-leaf to 2-leaf stage, mostly 1 plumule 37 to 62 mm, secondary roots starting on few.....	Oct. 16-22.....	8.0	11.0	5.0	25	0
Oct. 23	20	2-leaf stage.....	Oct. 23-29.....	6.4	9.1	3.8	10	42.9
Oct. 30	27	3-leaf stage.....	Oct. 30-Nov. 5.....	3.0	4.5	1.5	0.75	0
Nov. 6	34	Coleoptile loosening and tillers appearing in about half the plants.....	Nov. 6-12.....	3.4	6.2	.6	0.77	.8
Nov. 13	41	4-leaf stage; 1 to 2 tillers appearing in all plants.....	Nov. 13-19.....	4.8	6.6	3.0	0.76	.5
Nov. 20	48	do.....	Nov. 20-26.....	2.6	3.5	1.7	0.95	0
Nov. 27	55	do.....	Nov. 27-Dec. 3.....	.3	.9	-.3	0.95	0
Dec. 4	63	do.....	Dec. 4-10.....	-.3	.6	-1.1	0.95	0

\* Transplantings were continued weekly from Dec. 5 to Apr. 4, with the following results: Seedlings transplanted from noninfested to infested soil did not show any rosette, whereas those transplanted from infested to noninfested soil consistently showed 95 per cent rosetted plants.

#### EFFECTS ON DISEASE DEVELOPMENT

In the series in which the plants originally growing in the infested soil were transplanted to the disease-free soil, no rosette occurred in any of the first four transplantings. The absence of symptoms in the

plants which grew in infested soil for as long as 13 days from the date of seeding was very striking. When transplanted, the seedlings were in the 1-leaf to 2-leaf stage, and secondary roots were starting on a few. Certainly the soil temperature was favorable during this period, and the duration of exposure to infection could hardly explain the early lag and the sudden rise in the disease curve. The type of disease curve obtained suggests the possibility that the effect of the mercury in the 1:1,000 bichloride-of-mercury solution used in the surface sterilization of the underground parts may be responsible for this relation. Considering the disease curve further, it is seen that approximately 43 per cent of the plants were rosetted in the next weekly transplanting, October 23. The percentages increased to

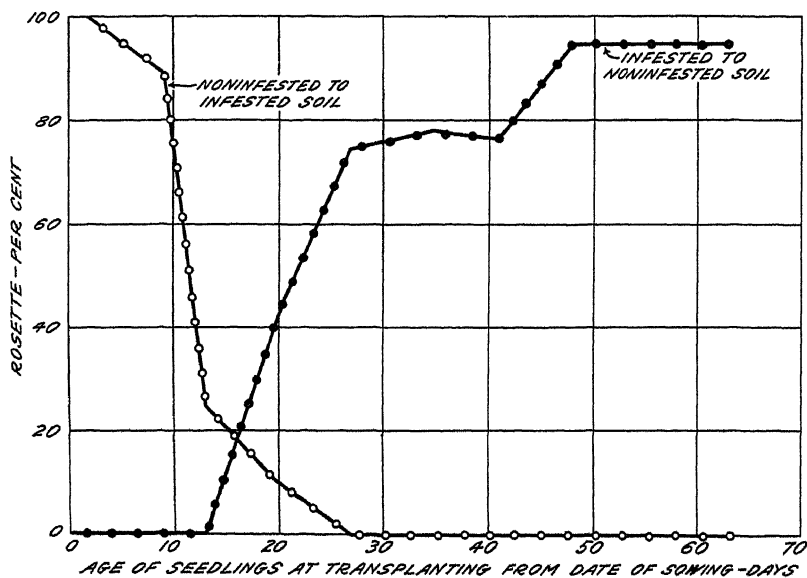


FIG. 4.—Influence of the duration of exposure to infection and of the stage of seedling growth on the development of the mosaic disease in Harvest Queen wheat under field conditions, as expressed in terms of rosette in seedlings made on October 3, 1923, a date suitable for field sowing, when seedlings were transplanted to or from infested soil at different intervals

from 75 to 80 per cent in the next three weekly transplantings, but it was not until the November 20 transplanting, or after a period of 48 days' exposure to infested soil, that the maximum percentage of the plants, 95 per cent, showed the rosetted condition. In all transplantings after this date, 95 per cent of the plants consistently showed the rosetted condition. In no case did any of the plants in the control transplantings show symptoms of rosette. (See fig. 5.)

For the series involving transplantings in the opposite direction, namely, from disease-free or control soil to infested soil, it is seen that the disease curve is a rather steep one. For the first transplanting, consisting of swollen kernels, the rosetted plants represent 100 per cent of the total. They decrease in percentage slightly in the next two transplantings, and decline rapidly to 25 per cent in the fourth (October 16) transplanting. In the October 23 transplanting, with seedlings in the 2-leaf stage, only 10 per cent of the plants developed rosette, and in the October 30 transplanting, with seedlings in the

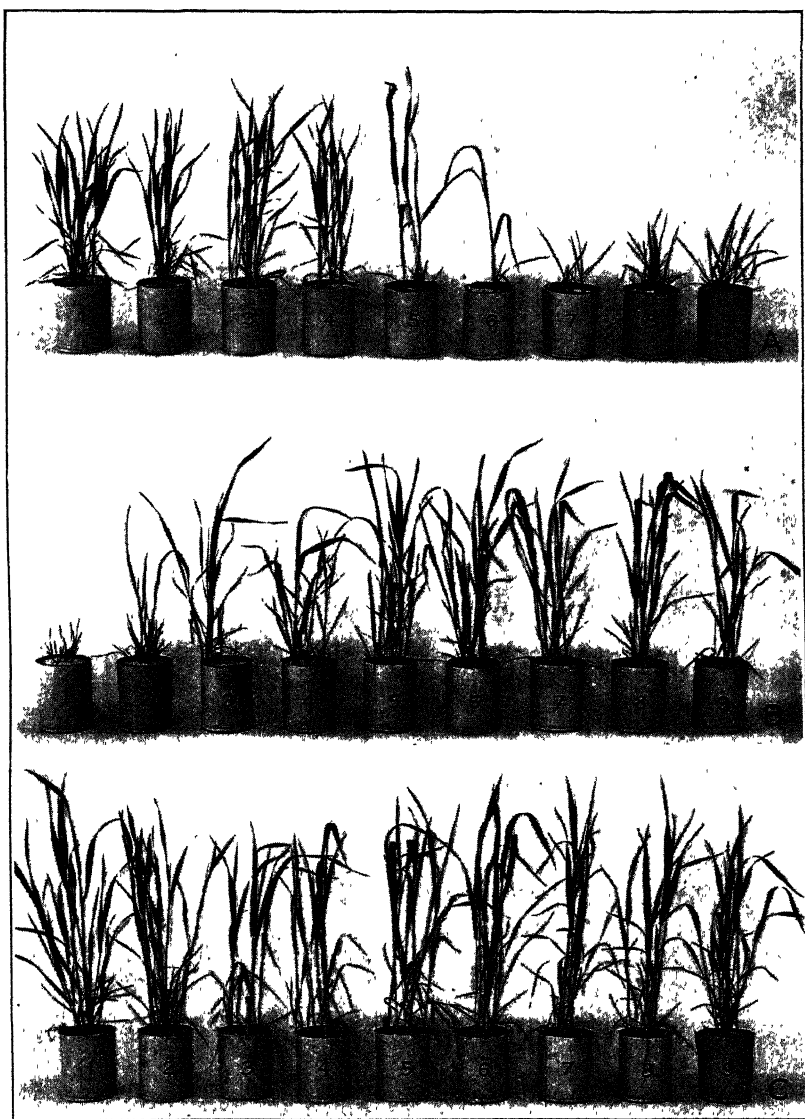


FIG. 5.—Harvest Queen wheat plants from the 1923-24 transplanting experiment, the data for which are given in Table 3: A, Seedlings transplanted from infested to noninfested soil; B, seedlings transplanted from noninfested to infested soil; C, seedlings transplanted from noninfested to noninfested soil (controls). In each of the corresponding series, A, B, and C, the transplantings were all made on the following dates, respectively: No. 1, October 5; No. 2, October 8; No. 3, October 12; No. 4, October 16; No. 5, October 23; No. 6, October 30; No. 7, November 6; No. 8, November 13; No. 9, November 20. Note the increasing appearance of rosette in the late transplantings in series A, and in the early transplantings in series B, and also the absence of rosette in the controls, series C. (Photographed by H. H. McKinney)

3-leaf stage, none of the plants showed rosette. No indications of rosette occurred in any of the subsequent transplantings. (See fig. 5.) It must be remembered, however, that the mean weekly soil temperature was gradually declining and at the last-mentioned date of transplanting it was near the point where growth activities cease. There-

fore the curve for the disease development (fig. 4) can not be analyzed too critically, for, as the seedlings were becoming older with each successive transplanting, the soil temperature at the same time was gradually declining. Two variable factors thus were involved, namely, seedling age and soil temperature, both of which are important and each of which influences and possibly may obscure the effect of the other.

#### FIELD TRANSPLANTING EXPERIMENTS, 1924-25

The experiment conducted during 1924-25 was a continuation and expansion of that of 1923-24 and was designed to permit analysis of the individual effects of certain factors which were operating together in the previous experiment. These factors include the period of exposure to infection and the root sterilization with  $\text{HgCl}_2$ , on the one hand, and the stage of seedling development and the environmental conditions, on the other. Both the Harvest Queen and the Currell varieties were used in this experiment.

Beginning August 4, seedlings of each variety were made at weekly intervals until November 11, in infested and in disease-free soil. The grain was sown in 10-inch earthen pots, the pots were sunk in the ground after planting, and the seedlings were transplanted as indicated. The seedlings were watered periodically before and after transplanting. Four transplantings were made, September 16, October 7, October 27, and November 18, respectively. Each transplanting involved seedlings of 6 different ages, expressed in weeks, namely, 6, 5, 4, 3, 2, and 1. The methods employed and the number of seedlings transplanted from disease-free control soil to infested soil, and vice versa, were the same as in the previous experiment. The seedlings growing originally in infested soil were transplanted both with and without the surface sterilization of the underground parts for 5 minutes in 1:1,000 bichloride of mercury. Approximately 4,000 seedlings were involved in this transplanting experiment.

#### EFFECTS ON HOST DEVELOPMENTS

The results of this experiment together with the soil-temperature data, are presented in Tables 4 and 5. The development of the seedlings of corresponding ages varied somewhat at the different transplantings. In general, however, the 1-week, 2-week, and 3-week seedlings in each of the four series represented 1-leaf, 2-leaf, and 3-leaf stages, respectively, with only the seminal roots developed. The seedlings in the late series were somewhat smaller than the corresponding ones in the earlier series. The 5-week and 6-week plants of the first series produced a very rank top growth on which leaf rust was prevalent. As a result of the severe rust infection and the high soil and air temperatures, these plants were pale green. The leaves in many cases were yellow, and the older ones were dead or dying. The other seedlings of this series were dark green, vigorous, and robust. The seedlings of the successive series presented general growth relations similar to those for the first series. The rust infection became less on the old plants of the next two transplantings and was absent in the last one. The top growth of the corresponding seedlings decreased more or less with the successive transplantings and the root systems became relatively larger. *Helminthosporium* infection was

especially pronounced on the basal structures of plants growing in infested soil and developing at relatively high soil temperatures.

Considerable variation occurred in the development of tillers and subcoronal internodes in each variety; but, on the whole, Harvest Queen showed a tendency to produce more tillers, and Currell showed a tendency to develop more pronounced subcoronal internodes. The Harvest Queen seedlings were very hardy and generally survived the severe winter conditions better than the Currell seedlings. In the case of both varieties, the percentage of sterilized seedlings which were winterkilled was greater than that of the unsterilized seedlings.

TABLE 4.—*Influence of the period of exposure to infection and of surface disinfection of transplanted seedlings, in relation to daily fluctuating soil temperatures, on the development of the mosaic disease in Harvest Queen and Currell wheats, as determined by transplanting seedlings of six different ages from naturally infested to noninfested soil on each of four different dates during the fall of 1924*

Dates of sowing	Average soil temperature in the week following sowing (° C.)			Time elapsed between sowing and emergence (days)	Period of exposure to infested soil (weeks)	Date of transplanting	Development of mosaic disease (per cent) in—					
	Mean	Maximum	Minimum				Harvest Queen				Currell	
							Mottling <sup>a</sup> when sterilized	Mottling <sup>a</sup> when not sterilized	Rosette when sterilized	Rosette when not sterilized	Mottling <sup>a</sup> when sterilized	Mottling <sup>a</sup> when not sterilized
Aug. 4 .....	22.9	26.4	19.5	4	6	Sept. 16	45 D	14 D	0	0	50.0 D	75.0 D
Aug. 11 .....	23.1	28.7	17.4	5	5		71 D	18 D	0	0	57.9 D	35.0 D
Aug. 18 .....	21.3	25.0	17.6	4	4		100 D	19 D	0	0	75.0 D	35.7 D
Aug. 25 .....	21.8	25.7	17.8	4	3		92 D	14 D	8.3	0	64.3 D	20.0 D
Sept. 1 .....	15.8	18.8	12.9	6	2		43 D	90 C	14.3	50.0	72.2 C	47.4 C
Sept. 8 .....	12.7	14.6	10.7	6	1		40 D	100 C	0	66.7	43.8 C	70.6 C
Aug. 25 .....	21.8	25.7	17.8	4	6	Oct. 7	71 C	40 D	42.9	40.0	37.5 C	25.0 D
Sept. 1 .....	15.8	18.8	12.9	6	5		50 C	91 C	50.0	63.6	72.2 C	64.7 C
Sept. 8 .....	12.7	14.6	10.7	6	4		100 C	100 C	( <sup>b</sup> )	83.3	85.7 C	94.1 C
Sept. 16 .....	14.8	18.0	11.5	5	3		67 C	37 C	20.0	70.2	66.7 C	46.7 F
Sept. 22 .....	13.3	16.3	10.4	6	2		92 C	71 C	30.8	57.1	43.8 M	41.2 M
Sept. 29 .....	10.7	12.8	8.5	6	1		44 D	30 D	0	0	31.3 D	25.0 D
Sept. 16 .....	14.8	18.0	11.5	5	6	Oct. 27	38 C	93 C	37.5	53.3	73.3 C	53.3 D
Sept. 22 .....	13.3	16.3	10.4	6	5		40 C	80 C	50.0	60.0	72.2 C	55.0 M
Sept. 29 .....	10.7	12.8	8.5	6	4		80 C	100 C	53.3	42.1	86.7 C	63.2 M
Oct. 7 .....	10.3	11.7	8.9	8	3		58 C	100 C	50.0	42.9	87.5 C	58.3 C
Oct. 13 .....	10.1	11.5	8.7	8	2		50 D	18 D	0	0	55.6 D	27.1 D
Oct. 20 .....	5.4	6.5	4.3	8	1		25 D	42 D	0	0	50 D	46.2 D
Oct. 7 .....	10.3	11.7	8.9	8	6	Nov. 18	0	100 C	0	0	100 C	50.0 C
Oct. 13 .....	10.1	11.5	8.7	8	5		0	73 D	0	0	33 C	37.5 C
Oct. 20 .....	5.4	6.5	4.3	8	4		( <sup>b</sup> )		( <sup>b</sup> )	( <sup>b</sup> )	( <sup>b</sup> )	( <sup>b</sup> )
Oct. 27 .....	6.1	7.8	4.4	7	3		0		0	( <sup>b</sup> )	( <sup>b</sup> )	( <sup>b</sup> )
Nov. 4 .....	3.1	4.7	1.6	8	2		( <sup>d</sup> )		0	( <sup>b</sup> )	( <sup>b</sup> )	( <sup>b</sup> )
Nov. 11 .....	2.0	2.9	1.1	( <sup>c</sup> )	( <sup>c</sup> )	( <sup>c</sup> )						

<sup>a</sup> Mottling symbols: D=Doubtful, F=faint, M=midmottley, C=conspicuous.

<sup>b</sup> All plants winterkilled after transplanting.

<sup>c</sup> Only a few plants survived, and the percentage was obtained by using the average of percentages from the next older and next younger seedlings.

<sup>d</sup> One plant survived and showed unmistakable mottling.

<sup>e</sup> All seedlings winterkilled before emergence.

#### EFFECTS ON DISEASE DEVELOPMENT

From the results of the experiments with the Harvest Queen seedlings subjected to the sterilization treatment, which are shown in Table 4 and graphically presented in Figure 6, it will be seen that the rosetted condition developed quite differently and that the interval of expo-

sure, the stage of seedling development at the time of transplanting, and the soil temperature were important factors influencing this condition. In the 6-week, 5-week, and 4-week seedlings transplanted September 16 it is interesting to note that no rosette occurred. The seedlings developed at mean weekly soil temperatures of  $21^{\circ}\text{C}$ . and above. Even the minimum soil temperatures of  $17^{\circ}$  to  $19^{\circ}\text{C}$ . for short periods each day were not sufficient to permit infection. These results agree well with those in the studies under controlled soil temperatures. The 3-week and 2-week transplanted seedlings showed only a small number of rosetted plants, 8.3 and 14.3 per cent, respectively, and the 1-week seedlings showed no cases of typical rosette. One plant, however, showed slightly suspicious indications.

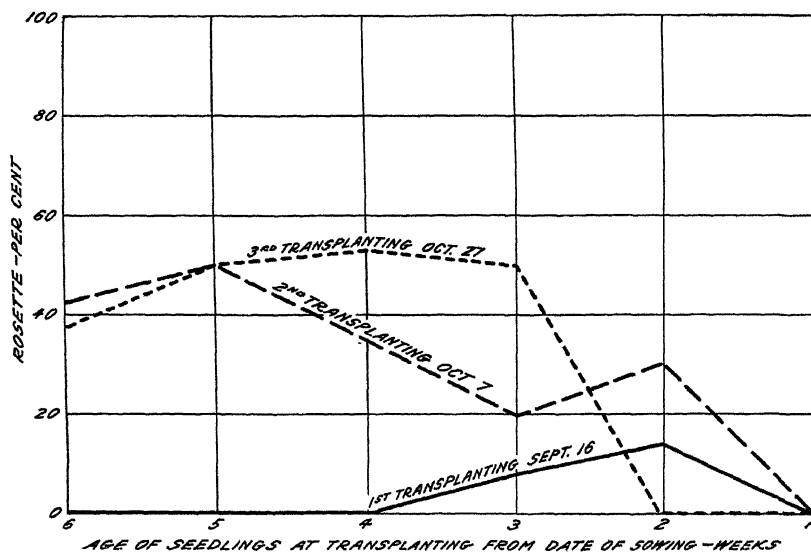


FIG. 6.—Influence of the period of exposure to infection on the development of rosette in Harvest Queen wheat under field conditions, as determined by transplanting seedlings of different ages from infested to noninfested soil on different dates during the fall of 1924. The surface of underground seedling parts were disinfested for 5 minutes in 1:1,000 bichloride of mercury immediately preceding transplanting.

In the second transplanting, October 7, all of the seedlings except the 1-week series showed rosette, and the percentages were higher than those of the corresponding seedlings of the first transplanting. The greatest number of diseased plants, 50 per cent, occurred in the 5-week stage, followed in order by the 6-week, 4-week, 2-week, and 3-week stages. The mean weekly soil temperatures ranged from  $10.3^{\circ}$  to  $2^{\circ}\text{C}$ .

The third series, transplanted October 27, showed almost 50 per cent of rosette in the 5-week, 4-week, and 3-week seedlings. The 6-week seedlings had slightly fewer diseased plants than the above mentioned seedlings. It is of interest to note that neither the 2-week nor the 1-week seedlings showed definite rosette. In the 2-week stage 10 per cent of the seedlings looked a little questionable, but none of the 1-week seedlings showed any indications of rosette. These seedlings, it must be remembered, developed at lower soil temperatures than the older seedlings of the series, and furthermore their time interval of exposure to infection was much shorter.

The rosette data for similar seedlings not receiving the surface sterilization of underground parts also are given in Table 4 and the data are presented as curves in Figure 7. In general, the percentages of rosetted plants are higher in the unsterilized seedlings than in the sterilized seedlings, especially in the case of the younger ones. These results indicate that the  $\text{HgCl}_2$  sterilization either is destroying or inactivating the causal agent in some way or is interfering with its subsequent development or activity in the plant. This, then, would tend to explain the lag in the early part of the disease curve for the 1923-24 experiments. The sterilized and unsterilized plants showed no visible variations in their development, and in view of this similarity there is a suggestion that the  $\text{HgCl}_2$  possibly may be exerting its effect more on the causal agent than on the host.

In the first transplanting, September 6, the 6-week, 5-week, 4-week, and 3-week seedlings did not show any signs of the rosetted condition.

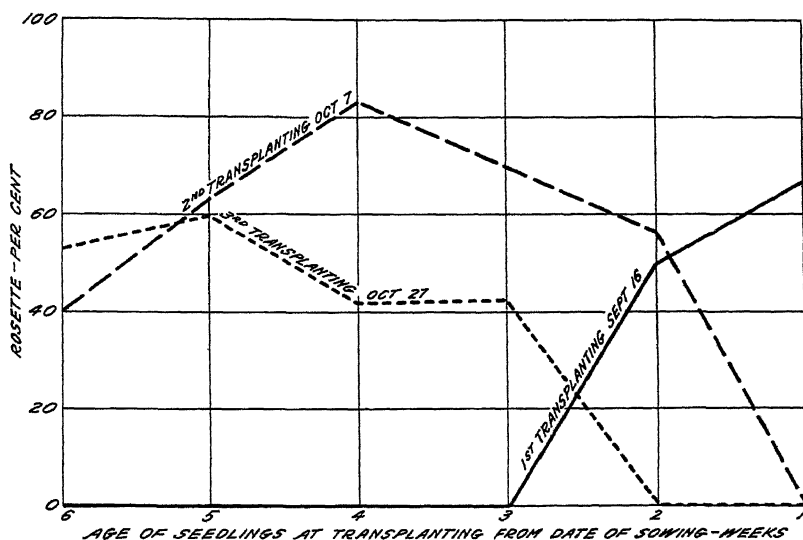


FIG. 7.—Influence of the period of exposure to infection on the development of rosette in Harvest Queen wheat under field conditions, as determined by transplanting seedlings of different ages from infested to noninfested soil on different dates during the fall of 1924, when no surface disinfection of underground parts was given

These results agree well with the soil-temperature relations previously considered. The relatively large number of rosetted plants occurring in the 1-week and 2-week seedlings, 67 and 50 per cent, respectively, is very striking, as compared with the values for the corresponding sterilized seedlings, 0 and 14 per cent, respectively. Even though the 2-week seedlings were exposed to infested soil one week longer than the 1-week seedlings, the latter had a higher percentage of disease. An examination of the soil-temperature data, shows that the 1-week seedlings experienced a slightly lower and more favorable soil temperature than the 2-week seedlings. Under these conditions it appears that the soil-temperature factor is more important than the period of exposure. It appears further that, under favorable conditions, an extremely high percentage of the disease may develop when the seedlings have grown in infested soil for a period of only one week from date of seeding.

The plants from the next transplanting, October 7, show high percentages of disease in all cases except the 1-week seedlings. The highest percentage, 83, occurred in the 4-week stage, followed closely by the 3-week, 5-week, and 2-week stages. A considerable decrease occurred in the 6-week seedlings, and no indications of disease occurred in the 1-week seedlings.

In the third transplanting, October 27, very similar effects were obtained in the 6-week, 5-week, 4-week, and 3-week seedlings, namely, 42 to 60 per cent. Neither the 1-week nor 2-week seedlings showed any indications of disease.

In the fourth transplanting, November 18, no indications of rosette were observed, although only a relatively few seedlings survived the winter.

A noteworthy point is brought out by results of the experiment with the seedlings of the October 7 sowing used in the third and the fourth transplantings. From Table 4 it will be seen that 43 to 50 per cent of the plants in the third transplanting showed rosette, whereas plants from the same sowing in the fourth transplanting did not show the disease. Only nine plants of the last transplanting survived the winter, so that the number is too small to permit of any conclusions. However, it appears that either the infected plants were more easily winter injured and that all were killed, or else that the development of the disease was interfered with in some way by the transplanting of the host at the extremely late date, November 18. In this connection it may be said that regardless of the increased period of exposure to infested soil, the oldest seedlings of a particular transplanting did not show the maximum development of rosette. Younger seedlings exposed to infection for even shorter time intervals had higher percentages of rosette. The age of the seedling at the time of transplanting, therefore, appears to be a contributing factor.

Thus far, only the rosetted condition of Harvest Queen seedlings growing in infested soil has been considered. The mottling phase likewise has been studied in all cases. But as pointed out earlier, the leaf mottling is a very variable manifestation and one extremely difficult to express. The mottling data for the Harvest Queen seedlings growing in infested soil are included also in Table 4, but no disease curves are presented.

In both the sterilized and unsterilized transplanted seedlings, it may be said that variable leaf mottling generally occurred throughout the entire experiment. The mottling was so faint as to be questionable in most cases on the older seedlings of the first transplanting, and high percentages of conspicuous mottling occurred only in the 1-week and 2-week unsterilized seedlings. This is the only outstanding instance in which the surface sterilization of the seedlings appears to have interfered with the mottling development somewhat as it appeared to do with the rosette development. Conspicuous mottling generally was obtained in the subsequent transplantings, with the exception of somewhat consistently questionable mottling in the youngest transplanted seedlings. In cases in which the mottling was so faint as frequently to be questionable, no rosette occurred. Unsterilized seedlings of the fourth transplanting showed excellent mottling in one case and questionable mottling in the other. No cases of rosette occurred in this transplanting. It appears, therefore,

that mottling may occur under a wider range of conditions than rosette.

While there were no consistent differences in the mottling of the sterilized and unsterilized series, the mottling curves for the different transplantings appeared to be more consistent and uniform in the case of the sterilized seedlings than in the case of the unsterilized seedlings. Reference to Table 4 will reveal the mottling data for the Currell variety. They are very similar to those just considered for the Harvest Queen variety and need not be considered further. It should be pointed out, however, that conspicuous mottling in varying percentages was obtained in the 1-week and 2-week seedlings in both the sterilized and unsterilized series of the first transplanting and in all the surviving seedlings of the last transplanting.

Simultaneously with the different transplantings of seedlings from infested soil to disease-free soil, corresponding transplantings of both

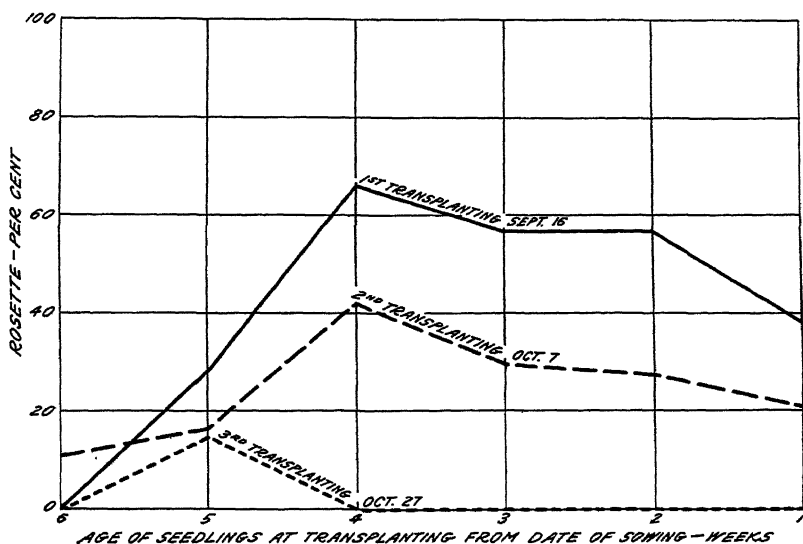


FIG. 8.—Influence of stage of seedling growth on the development of rosette in Harvest Queen wheat under field conditions, as determined by transplanting seedlings of different ages from noninfested to infested soil on different dates during the fall of 1924

varieties were made in the opposite direction. The results of this study are included in Table 5 and the curves appear in Figure 8.

If the rosette phase in the Harvest Queen variety is considered first, it will be seen that the disease generally developed to the greatest extent in the different seedlings of the first transplanting (September 16), followed uniformly by those of the second transplanting (October 7). The curves are remarkably uniform and consistent. In fact, the only noticeable difference is that, whereas no rosette developed in the 6-week seedlings of the first transplanting, a relatively small percentage developed in the corresponding seedlings of the second transplanting.

The disease curves further demonstrate the influence of soil temperature, and agree well with the results obtained in the controlled and uncontrolled soil-temperature experiments. While age of seedling and soil temperature are important factors influencing infection,

neither apparently modifies or obscures the effect of the other under the experimental conditions employed. In both the first and second transplantings, the seedlings 4 weeks old at the time of transplanting showed the highest percentage of rosette, followed in order by the 3-week, 2-week, 1-week, 5-week, and 6-week stages.

TABLE 5.—*Influence of stage of seedling development, in relation to daily fluctuating soil temperatures, on the development of the mosaic disease of Harvest Queen and Currell wheats, as determined by transplanting seedlings of six different ages from noninfested to naturally infested soil, on each of four different dates, during the fall of 1924*

Date of sowing	Age of seedlings when transplanted	Date of transplanting	Average weekly soil temperatures for six successive weeks after transplanting				Development of mosaic disease (per cent) in—		
			Period	Mean	Maximum	Minimum	Harvest Queen		Currell, mottling
							Ro-sette	Mot-tling	
	Weeks			° C.	° C.	° C.			
Aug. 4.....	6	Sept 16	Sept. 15-21.....	14.8	18.0	11.5	0	<sup>a</sup> 8.0	46.2
Aug. 11.....	5		Sept. 22-28.....	13.3	16.3	10.4	28.6	71.0	50.0
Aug. 18.....	4		Sept. 29-Oct. 5.....	10.7	12.8	8.5	66.7	67.0	50.0
Aug. 25.....	3		Oct. 6-12.....	10.3	11.7	8.9	57.1	71.0	55.0
Sept. 1.....	2		Oct. 13-19.....	10.1	11.5	8.7	57.1	50.0	11.8
Sept. 8.....	1		Oct. 20-26.....	5.4	6.5	4.3	38.1	10.0	17.6
Aug. 25.....	6	Oct 7	Oct. 6-12.....	10.3	11.7	8.9	11.1	33.0	33.3
Sept. 1.....	5		Oct. 13-19.....	10.1	11.5	8.7	16.7	60.0	31.6
Sept. 8.....	4		Oct. 20-26.....	5.4	6.5	4.3	42.1	68.0	41.2
Sept. 16.....	3		Oct. 27-Nov. 2.....	6.1	7.8	4.4	30.1	80.0	35.0
Sept. 22.....	2		Nov. 3-9.....	3.1	4.7	1.6	27.8	50.0	5.0
Sept. 29.....	1		Nov. 10-16.....	2.0	2.9	1.1	21.1	53.0	24.0
Sept. 16.....	6	Oct. 28 <sup>b</sup>	Oct. 27-Nov. 2.....	6.1	7.8	4.4	0	15.0	<sup>c</sup> 10.0
Sept. 22.....	5		Nov. 3-9.....	3.1	4.7	1.6	15.0	40.0	10.5
Sept. 29.....	4		Nov. 10-16.....	2.0	2.9	1.1	0	10.0	20.0
Oct. 7.....	3		Nov. 17-23.....	.1	.9	—	0	0	13.0
Oct. 13.....	2		Nov. 24-30.....	-3.5	-2.8	-4.2	0	<sup>a</sup> 5.0	20.0
Oct. 20.....	1		Dec. 1-7.....	( <sup>d</sup> )	( <sup>d</sup> )	( <sup>d</sup> )	0	0	0
Oct. 7.....	6	Nov. 18 <sup>b</sup>	Nov. 17-23.....	.1	.9	—	0	<sup>a</sup> 6.0	( <sup>e</sup> )
Oct. 13.....	5		Nov. 24-30.....	-3.5	-2.8	-4.2	0	0	( <sup>e</sup> )
Oct. 20.....	4		Dec. 1-7.....	( <sup>d</sup> )	( <sup>d</sup> )	( <sup>d</sup> )	0	0	( <sup>f</sup> )
Oct. 27.....	3		Dec. 8-14.....	( <sup>d</sup> )	( <sup>d</sup> )	( <sup>d</sup> )	( <sup>e</sup> )	( <sup>e</sup> )	( <sup>f</sup> )
Nov. 3.....	2		Dec. 15-21.....	( <sup>d</sup> )	( <sup>d</sup> )	( <sup>d</sup> )	( <sup>e</sup> )	( <sup>e</sup> )	( <sup>f</sup> )
Nov. 11.....	1		Dec. 22-28.....	( <sup>d</sup> )	( <sup>d</sup> )	( <sup>d</sup> )	( <sup>e</sup> )	( <sup>e</sup> )	( <sup>f</sup> )

<sup>a</sup> Mottling so faint as to be questionable.

<sup>b</sup> The regular date of transplanting would have been a day earlier, and therefore the temperature record for the week would have been begun one day earlier than the date of transplanting.

<sup>c</sup> Mottling faint.

<sup>d</sup> Soil thermograph out of order.

<sup>e</sup> Plants winterkilled after transplanting.

<sup>f</sup> Plants killed before emerging.

Whether the 4-week seedlings actually are more susceptible to infection than the seedlings of other ages, or merely represent the best stage for transplanting, is not known. The fact that in only one instance a small percentage of rosette developed in the transplanted 6-week seedlings indicates that little or no infection is likely after a period of 5 or 6 weeks from date of seeding. The decline in the rosette curve with the 1-week seedlings very probably is due to the diminution of surface exposed to the infested soil, caused by the smaller root system. In addition, the setback of transplanting very probably is greater with these than with older seedlings. However, no perceptible retardation was noted.

In the third transplanting, the only cases of rosette occurred in the 5-week seedlings, 15 per cent showing the disease. The mean weekly soil temperature for the five successive weeks after transplanting ranged from  $6.1^{\circ}$  to  $-3.5^{\circ}$  C. The fact, then, that the soil temperature was so relatively low and that the transplanted seedlings grew under such unfavorable conditions, probably explains the absence of the disease.

In the fourth transplanting, seedlings of the three latest sowings were winterkilled soon after emergence. A few plants of the 6-week, 5-week, and 4-week seedlings survived, but none of these showed any indications of rosette. The soil temperature ranged from  $0.1^{\circ}$  C. to considerably below  $-4.2^{\circ}$  C. after the seedlings had been transplanted to infested soil.

From an examination of the mottling data for the Harvest Queen variety in Table 5, it is evident that mottling occurred in each of the different ages of seedlings of the first two transplantings. The two disease curves, though not shown in this paper, are very similar, the highest percentages of mottled plants occurring in the 3-week, 4-week, and 5-week seedlings and diminishing in younger or older seedlings. The mottling was questionable in the oldest seedlings of the first transplanting and it was either absent or very faint in the younger seedlings of the third transplanting.

It is interesting to note that a small percentage of faint mottling occurred in the oldest seedlings of the fourth transplanting, even though the soil temperature ranged from  $0.9^{\circ}$  C. to below  $0^{\circ}$  C. after the transplanting to infested soil.

In general, the percentages of plants showing mottling were higher than those showing rosette, and in cases in which the mottling was either absent or faint no rosette occurred. It thus appears that the leaf-mottling condition is capable of developing over a wider range of both soil temperatures and seedling ages than is the rosette condition.

The leaf-mottling data for the Currell variety also are shown in Table 5. Higher percentages of mottling occurred in the seedlings of the first transplanting, followed in order by those of the second and third transplantings. Mottling was very uniform with the 3-week, 4-week, and 5-week seedlings in each transplanting, and a noticeable decrease appeared in the younger seedlings. No mottling was evident in the 1-week seedlings of the third transplanting. All seedlings of the fourth transplanting were winterkilled, and no mottling data were obtainable. In comparing the leaf-mottling data of Currell with those of Harvest Queen, it is evident that the percentages generally are less and that the disease curves, though not shown in this paper, differ somewhat. Nevertheless, the soil-temperature and seedling-stage ranges are about the same for each variety.

#### DISCUSSION

In diagnosing the mosaic disease of winter-wheat seedlings, macroscopical symptoms have been employed as criteria. Microscopical symptoms, such as intracellular bodies, offer a more delicate diagnosis in extreme or doubtful cases, but the necessity of studying large numbers of plants makes this method slow and laborious. Until the presence of a causal agent can be detected, the mosaic disease

of wheat, like all other virus diseases, must be defined in terms of host response. Obviously, the presence of symptoms always indicates that infection has occurred, but the absence of such symptoms does not necessarily indicate that infection has not occurred.

As a rule, the rosette phase is a generally constant and unmistakable index, and its occurrence can be effectively expressed in terms of percentages. Intermediate stages may occur in some instances, but they have been the exception in the experiments reported. The leaf mottling, on the other hand, is a much more difficult response to calibrate, inasmuch as both the percentage of plants showing mottling and the intensity of the mottling are variables. The present-day quantitative and qualitative methods of mottling determination, therefore, are unsatisfactory and inadequate, and this condition serves as the explanation for the greater emphasis given to the rosette phase than to the mottling phase throughout this paper.

Winter-wheat seedlings are particularly hardy and may withstand much rough treatment with no visible injury. These properties, therefore, well adapt them for experimentation of this type. The method of transplanting, of course, is open to criticism, for it necessitates the removal of the seedlings from the soil and causes considerable disturbance. However, at the time of these experiments, this method was the most natural and the only one applicable to the problem. It is realized that growth, or the ability of the tissues to resume growth after transplanting, is an important matter and that all factors governing growth before and after transplanting exert important influences. The transplanting results, therefore, must be accepted with these points in mind.

In general, the results presented in this paper indicate that the seedling infection and symptom expression of the mosaic disease in the Harvest Queen and Currell varieties are most pronounced under those environmental conditions which are best adapted for the development of the host during the seedling stages. Conditions, therefore, which promote high resistance to many plant diseases promote high susceptibility to the wheat mosaic disease. In this respect the wheat disease agrees well with the other known virus diseases of the mosaic type, as it has long been known and well established that the mosaic diseases occur and develop best with the most active host development. Similar relations, moreover, recently have been reported by Keitt (7), by Jones (6), and by Fellows (2), for certain plant diseases outside of the virus category.

The data herein presented certainly demonstrate that soil temperature and soil moisture are important factors influencing infection and subsequent development of the mosaic disease of winter wheat. The fact that the disease developed only at low soil temperatures and decidedly best at high soil moistures is particularly significant. No doubt the effect of one or both of these two environmental factors is important in the frequent development of the disease in patchy and more or less circular areas under field conditions. However, the possible variation of soil infestation is a question that always must be considered.

The absence of rosette and mottling in seedlings developed at the relatively high soil temperatures of 23° and 30° C. and at low soil moistures brings up interesting questions at this time. What is the

explanation of these relations? Is it a question of either host response or virus activity, or both? Or, if it is purely a question of host response, is the resistance which is developed during an early seedling stage dominant throughout the susceptible seedling stage? Unfortunately, the information now available concerns only the disease expressed in terms of host response. Nothing is known concerning the virus response, and until data are obtained from property studies made on the virus in the soil, in the expressed tissue fluids, and isolated from these respective media, little can be offered in the way of a satisfactory explanation.

Different types of metabolism within the host occur at the low and the high soil-temperature extremes. These relations are very significant and undoubtedly play an important part in infection and the subsequent development of this disease. At the low temperatures, emergence is relatively slow, the roots develop first and faster than the plumules, root development is highly stimulated, the roots are fleshy in texture, the elongation of the subcoronal internode is inhibited, and the coleoptile develops faster than the growing culm, thereby protecting the growing tip. It is in the seedlings of this type and under the corresponding environmental conditions essential for their development that the mosaic disease of wheat develops best. Either the protection offered to the growing point by the coleoptile possesses no value in the case of this disease, contrary to its rôle in certain other soil-borne diseases, or else no infection occurs through this structure. Conversely, at the higher temperatures, emergence is relatively speedy, the plumules develop first and faster than the roots, root development generally is depressed, the roots are fine and fibrous, brown in color, and not fleshy in texture, the subcoronal internode is greatly stimulated, and the growing culm generally ruptures the coleoptile before emergence. It is in the seedlings of this type, and under the corresponding environmental conditions essential for their development, that the mosaic disease of wheat fails to develop.

From the standpoint of the causal agent, the results would seem to indicate that the absence of rosette and mottling at the higher soil temperatures of 23° C. or above was not due entirely to the temperature effect on the virus. If the virus is as sensitive as the most sensitive virus known to-day, namely, the virus causing the cucumber mosaic, it would be able to withstand temperatures approaching 50° to 60° C. for short periods. However, too much emphasis should not be placed on a comparison of the results of such property studies; for, unless the experiments are conducted under the same or similar conditions and in the same or similar medium, the results possess little in the way of significance.

It is difficult also to believe that the absence of rosette and mottling can be explained entirely on the basis of a desiccating effect on the virus at the low soil moistures under favorable temperatures. Infested soil from Granite City, Ill., has been stored in a highly desiccated state over a period of four years and when sown to wheat has produced rosette and mottling most generally and severely.

While only the questions of host and causal agent have been considered thus far, it is possible also that some soil vector of an animal nature is active in the promotion of mosaic disease and that the different results may be due to the various responses of the disease

to the experimental conditions. So far, however, there is no evidence of such a vector.

Because the rosette phase of the mosaic disease of winter wheat is controlled completely by the use of resistant and immune varieties, as shown in previous papers (3, 8, 13), no recommendation for the control of the disease on a basis of date of seeding is made. In extremely early and late fall sowings, in cases where the disease is partially or completely controlled, the seeding dates are so far removed from the normal seeding dates in the field that they would be impracticable.

#### SUMMARY

The occurrence and subsequent development of the mosaic disease of winter wheat is closely associated with normally active growth of the seedling.

The period of infection is limited to the early seedling stage. Under favorable environmental conditions, an interval of one week from date of seeding is sufficient to permit a high percentage of rosetted or mottled plants. Where the environmental conditions were less favorable, mottling occurred at time intervals too short for the development of rosette.

The disease developed only within a relatively restricted soil-temperature range. In a test at the four different constant soil temperatures, 10°, 16°, 23°, and 30° C., the disease occurred only at 10° and 16° C. The leaf mottling of the Currell variety occurred to about an equal degree at each of the two soil temperatures, while the rosette and the leaf mottling of the Harvest Queen variety developed best at 16° C. No indications of either rosette or mottling appeared in either variety at the two higher soil temperatures.

A high soil-moisture content decidedly favored the occurrence of both rosette and leaf mottling, whereas a medium soil-moisture content was proportionately less favorable. No indication of symptoms appeared under the low soil-moisture contents.

Under the controlled conditions described, no shifts occurred in the soil-temperature and soil-moisture optima when the value for either factor was altered.

Rosette developed under field conditions at soil temperatures strikingly similar to those found most favorable in the controlled tanks. Faint mottling of both varieties, however, developed at somewhat higher soil temperatures under these fluctuating conditions.

In the case of seedlings transplanted from infested soil to uninfested soil, the age of the seedling at the time of transplanting appeared to be a factor influencing the development of the disease; that is, the percentage of diseased plants increased only within limits for increased periods of exposure to infested soil.

The rosette-disease infection curves for the seedlings of six different ages varied with the transplantings on different dates. These relations are in direct line with those obtained in the tank and the field soil-temperature experiments, the disease developing only at or below 16° C.

The susceptibility of the seedlings to rosette is influenced by the stage of seedling development. In general, the 4-week seedlings appeared most susceptible, followed in order by the 3-week, 2-week, 1-week, 5-week, and 6-week seedlings, respectively. Only in one

instance was the disease obtained in the 6-week seedlings, and the percentage then was very small.

For both varieties tested, mottling was obtained in each of the six different ages of seedlings. In contrast to rosette, the mottling appeared prominently in the 6-week seedlings.

Comparing the different varietal responses, it is evident that mottling was more general than rosette, and that the Harvest Queen variety showed higher percentages of mottled plants than the Currell variety.

Surface sterilization of the subterranean parts of infected seedlings with bichloride of mercury at the time of transplanting noticeably reduced the occurrence of rosette, especially in the younger seedlings. This treatment entirely prevented the occurrence of rosette in the 1-week seedlings.

Leaf mottling was not similarly affected by the surface sterilization. On the contrary, the percentages of mottling frequently were higher and noticeably more uniform in the sterilized than in the unsterilized series.

The evidence obtained does not throw any definite light on the nature of the causal agent.

Any possible causal relation of *Helminthosporium sativum* naturally occurring in the infested soil to either the rosette or mottling phases of this disease is further disproved.

No effective control measures for this disease are practicable on the basis of seeding date, as suggested by the soil-temperature relations.

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# FACTORS AFFECTING THE POPPING QUALITY OF POP CORN<sup>1</sup>

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## INTRODUCTION

Only during the last half century has corn popping developed into an industry of commercial importance. The resulting demand for pop corn has given the stimulus necessary to the growing of the crop in a large way and has expanded the acreage very considerably.

The quality of pop corn depends upon its palatability or flavor and upon its popping expansion. Although it is commonly recognized that great differences exist in the "poppability" of different varieties and in various lots of the same variety, the matter seems not to have been studied in a critical way.

All starchy corns may be placed in one of the four classes—pop corn, flint corn, dent corn, or flour corn—on the basis of

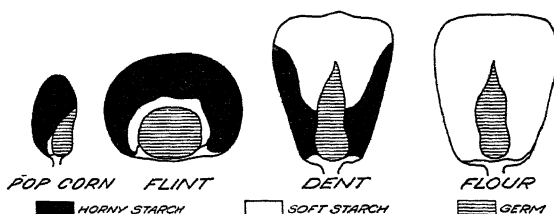


FIG. 1.—Diagrammatic representation of location and relative proportions of horny starch and soft starch in the four main classes of starchy corn

distribution and relative proportions of hard and soft starch in the endosperm. The endosperm of the best strains of pop corn is corneous throughout or contains only a small core of soft starch near the center. (Fig. 1.) In flint corn the endosperm consists of a small quantity of soft starch, near the embryo, completely surrounded by corneous starch. In dent corn the corneous starch is confined mainly to the sides of the kernels, soft starch constituting a larger proportion than in flint corn. In flour corn the endosperm is entirely of soft starch except for vestigial traces of hard starch.

The popping properties of the various types of corn follow rather closely the relative proportions of hard or corneous starch in the endosperm. Pop corn, with the greatest proportion of hard starch of any of the four types, is far better than the others in poppability. Flint corn may pop poorly to fairly well, depending on the strain. Dent corn rarely will pop at all, though occasionally a few kernels will pop feebly. Flour corn will not pop at all.

It is generally conceded that the popping process is due to the sudden liberation of pressure produced by steam generated within the kernel.<sup>2</sup> The source of the steam is the moisture contained in

<sup>1</sup> Received for publication July 11, 1927; issued November, 1927. This paper is a report of cooperative investigations made by the Bureau of Plant Industry, U. S. Department of Agriculture, and the Kansas Agricultural Experiment Station. Department of Agronomy contribution No. 169.

<sup>2</sup> WEATHERWAX, P. THE POPPING OF POP CORN. *Ind. Acad. Sci. Proc.* 1921: 149-153. 1922.

the kernel and perhaps to a limited extent, also, water of constitution produced by partial breaking down of the starch molecule when heated.<sup>3</sup> The confining structure, according to Weatherwax, is the colloidal matrix in which the starch grains are embedded within the cell. The success of the explosion in expanding the kernel and completely disrupting its original structure to produce a uniform and tender product depends upon three conditions: (1) Optimum moisture content, (2) an elastic and tenacious confining structure, and (3) proper application of heat.

The first and third of these conditions, viz, optimum moisture content and proper application of heat, may be controlled at the time of popping. The second condition, however, is a property of the endosperm and varies with the sample. It is believed that differences in the poppability of various strains are definitely inherited and that the properties of endosperm structure have a genetic factorial basis, although the mode of inheritance is undoubtedly complicated. As a preliminary step in testing the possibilities of pop corn improvement through breeding, this paper is concerned chiefly with a study of the degree of association between certain morphological kernel characters and popping yield.

#### EXPERIMENTAL METHODS

With the exception of tests covering the influence of moisture content on poppability, all of the corn used in these experiments was of uniform and probably nearly optimum moisture content. The popping trials were conducted at Washington, D. C., during the winter months. Until the time of popping the samples were stored in an outside shelter protected from direct precipitation but subject to continuous free circulation of air.

The popping trials were made in a wire popper over a gas burner. A wire gauze over the burner helped to give a more uniform distribution of heat. The flame was adjusted so that popping would commence in about one and one-half minutes, which time had previously been found to give the best results. A wire swing for the popper assured a constant distance between the corn and the source of heat.

The index of poppability used in this paper is the ratio of the volume after popping to the volume of the unpopped corn, and for convenience is termed "expansion." Glass graduates were used to measure volumes both before and after popping. A uniform sample 20 cubic centimeters in volume was used throughout for popping, and, except where otherwise stated, the expansion is based on duplicate determinations. The expansion was determined only to the nearest whole number. Besides expansion, six characters of the unpopped kernels were considered, viz, percentage of soft starch, weight of 100 kernels, size as measured inversely by the number of kernels in 25 cubic centimeters, length of kernels, breadth of kernels, and thickness of kernels.

The proportion of soft starch in the endosperm did not lend itself to any simple exact measurement and therefore was estimated. Ten random kernels from each ear were split lengthwise and the halves glued to cardboard. The percentage of soft starch then was esti-

<sup>3</sup> CARR, R. H., and RIPLEY, E. F. WHEAT PUTS "POP" IN POP CORN? *Ind. Acad. Sci. Proc.* 1920: 261-270, illus. 1921.

minated for each kernel separately, and the average of the 10 estimates was taken as the soft-starch content for the ear as a whole. The final ratings were not made until after a preliminary survey had given an intimate acquaintance with the material and had fixed the standards firmly in mind, so that it is thought that the estimates present a very fair basis for purposes of comparison.

Weight of kernels was obtained by weighing 100 kernels from each ear. Another measurement which is highly correlated negatively with weight was also taken, namely, the number of kernels in a measured volume of 25 cubic centimeters. Length of kernel was measured by laying 10 kernels end to end in a groove and dividing the total length in millimeters by 10. Breadth of kernel and thickness of kernel (from germinal to abgerminal face) were obtained similarly.

### INFLUENCE OF MOISTURE CONTENT ON POPPABILITY

It is well recognized that the moisture content of pop corn has an important influence on its poppability. Carr and Ripley,<sup>4</sup> however, state that within comparatively wide ranges the moisture content has little effect on popping expansion. Weatherwax,<sup>5</sup> taking a similar view, expresses the opinion that, although maximum, minimum, and optimum moisture contents are indicated, the range is wide. In an exhaustive study on the effect of moisture content and other factors on popping, Stewart<sup>6</sup> concludes that "a moisture content of 13 to 15 per cent appears to be the most favorable for maximum popping yield." He states also that, under New York conditions, outside storage from October to April will give a moisture content approaching the optimum.

TABLE 1.—*Expansion of successive 12-ear samples of White Rice pop corn taken while drying, showing relation between moisture content and poppability*

Sample No.	Moisture (per cent)	Expansion (vol-umes)	Sample No.	Moisture (per cent)	Expansion (vol-umes)	Sample No.	Moisture (per cent)	Expansion (vol-umes)
1.....	17.0	2	11.....	11.9	18	21.....	9.1	11
2.....	15.6	7	12.....	11.9	17	22.....	8.3	9
3.....	15.6	5	13.....	11.9	16	23.....	8.1	6
4.....	14.2	12	14.....	10.6	16	24.....	7.4	7
5.....	13.6	15	15.....	10.5	15	25.....	7.4	8
6.....	13.6	14	16.....	10.1	16	26.....	7.2	6
7.....	12.7	17	17.....	10.1	14	27.....	6.9	8
8.....	12.7	16	18.....	9.6	10	28.....	6.3	7
9.....	12.6	17	19.....	9.5	12			
10.....	12.6	16	20.....	9.2	11			

A stock of White Rice pop corn grown at Oconomowoc, Wis., was divided into a number of samples of 12 ears each, and successive moisture and popping determinations were made as the samples were drying. Each sample of 12 ears was shelled and thoroughly mixed before testing. The data for these tests are given in Table 1

<sup>4</sup> CARR, R. H., and RIPLEY, E. F. Op. cit.

<sup>5</sup> WEATHERWAX, P. Op. cit.

<sup>6</sup> STEWART, F. C. THE RELATION OF MOISTURE CONTENT AND CERTAIN OTHER FACTORS TO THE POPPING OF POP CORN. N. Y. State Agr. Expt. Sta. Bul. 505, 70 p., illus. 1923.

and are shown graphically in Figure 2. Each value for expansion is the mean of several determinations made from a given 12-ear sample. About 12 per cent appears to be the optimum moisture content for greatest expansion in this sample.

#### RELATION OF KERNEL CHARACTERS TO POPPABILITY IN WHITE RICE POP CORN

One hundred ears of White Rice pop corn grown, harvested, and cured under as nearly identical conditions as possible formed the material for a preliminary study of the relation of kernel characters to poppability. Data were obtained for the weight of 100 kernels, the number of kernels in a 25 cubic centimeter volume, the percentage

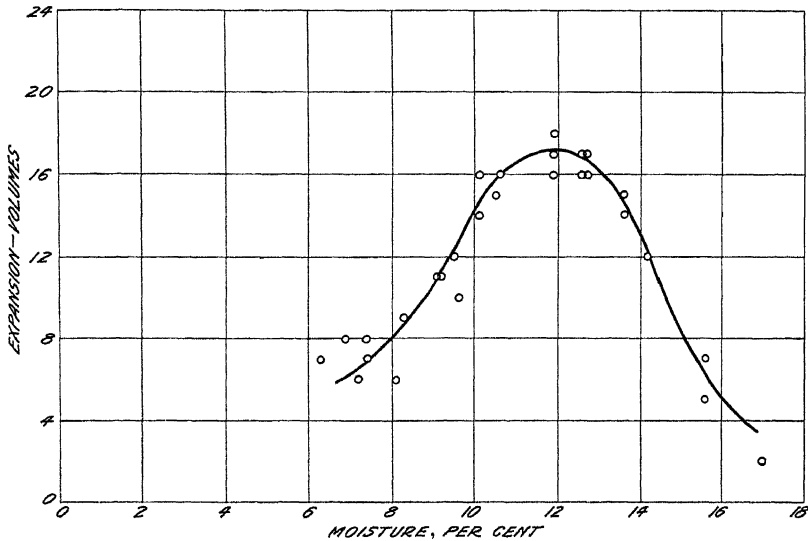


FIG. 2.—Relation between moisture content and popping expansion in White Rice pop corn

of soft starch, and the expansion for each ear. The expansion on popping ranged from 5 to 25 volumes. The ears were grouped into seven classes according to expansion, and the average obtained in each group for the various characters noted is shown in Table 2.

There is a distinct tendency for the size of the kernels to decrease as the expansion increases. In other words, the ears giving the greater increase in volume on popping tend on the average to have smaller kernels. This is true when kernel size is measured either by the weight of 100 kernels or by the number of kernels in 25 cubic centimeters. In group A the average expansion is 6.0, the average weight of 100 kernels is 15.5 gms., and the average number of kernels in 25 cubic centimeters is 130. At the other extreme, in group G, with an average expansion of 24.2, the weight of 100 kernels is only 11.6 gms. and the average number of kernels in 25 cubic centimeters is 177.4.

TABLE 2.—*Relation between certain kernel characters and popping quality of White Rice pop corn, as shown by the mean values of these characters when a population is grouped on the basis of expansion*[Correlation of expansion with weight of 100 kernels,  $-0.57 \pm 0.046$ , with number of kernels in 25 c. c.  $0.56 \pm 0.046$ ; and with per cent of soft starch,  $-0.55 \pm 0.047$ ]

Group	Range of expansion (volumes)	Number of ears	Average			
			Weight of 100 kernels (grams)	Number of kernels in 25 cubic centimeters	Percentage of soft starch	Expansion (volumes)
A-----	5-7	12	15.5	130	11.2	6.0
B-----	8-10	14	15.2	136	9.9	9.4
C-----	11-13	20	14.0	148	7.8	12.0
D-----	14-16	20	14.1	145	8.2	15.0
E-----	17-19	11	12.9	158	7.2	17.9
F-----	20-22	13	11.4	180	3.8	20.5
G-----	23-25	10	11.6	177	2.6	24.2

The relation of percentage of soft starch to poppability also is pronounced. Group A has an average soft-starch content of 11.2 per cent, whereas group G, with the largest popping expansion, has an average soft-starch content of only 2.6 per cent. There is a consistent decrease in the soft-starch content from group to group when the groups are arranged in ascending order on the basis of expansion.

The tendencies shown in these groupings suggest at once the probability of significant correlations between expansion and the three kernel characters measured. The correlation coefficients were calculated and are shown in Table 2. These coefficients are all about twelve times their probable errors and are of undoubted significance.

#### INFLUENCE OF KERNEL CHARACTERS ON POPPABILITY IN YELLOW PEARL POP CORN

In order to verify and amplify the results of the preliminary studies, a second experiment was planned, in which a larger number of ears of another variety was used, and the scope of the work was extended to include three additional kernel measurements. A sample of 394 ears of Yellow Pearl, which had uniform conditions for growth and curing, was used as the experimental material. The weight of 100 kernels, number of kernels in 25 cubic centimeters, percentage of soft starch, and expansion were determined as before. In an attempt to analyze further the relation between kernel size and expansion, the three additional measurements of length, breadth, and thickness of kernels were also taken.

TABLE 3.—*The means, standard deviations, and coefficients of variability of the various characters studied in a group of 394 ears of Yellow Pearl pop corn*

Symbol	Character	Mean	Standard deviation	Coefficient of variability
V-----	Expansion.....volumes..	24.20 $\pm$ 0.078	2.29 $\pm$ 0.055	9.45 $\pm$ 0.229
S-----	Percentage of soft starch.....	8.09 $\pm$ 0.139	4.09 $\pm$ 0.098	50.59 $\pm$ 1.495
W-----	Weight of 100 kernels.....grams..	15.05 $\pm$ 0.060	1.77 $\pm$ 0.043	11.78 $\pm$ 0.287
N-----	Number of kernels in 25 cubic centimeters..	143.10 $\pm$ 0.574	16.90 $\pm$ 0.406	11.81 $\pm$ 0.288
L-----	Length of kernel.....millimeters..	8.08 $\pm$ 0.015	.45 $\pm$ 0.011	5.52 $\pm$ 0.133
B-----	Breadth of kernel.....do....	5.70 $\pm$ 0.011	.33 $\pm$ 0.008	5.72 $\pm$ 0.138
T-----	Thickness of kernel.....do....	3.48 $\pm$ 0.009	.28 $\pm$ 0.007	8.01 $\pm$ 0.194

The ordinary statistical constants of the various measurements, together with the symbols used hereafter for the various characters, are shown in Table 3.

#### GROSS CORRELATIONS

The coefficients of correlation between expansion and each of the kernel characters and also those between the various combinations of kernel characters, are given in Table 4.

TABLE 4.—Gross correlations between all possible combinations of the various characters studied in a sample of 394 ears of Yellow Pearl pop corn

Character	Expansion (volumes) <i>V</i>	Percentage of soft starch <i>S</i>	Weight of 100 kernels (grams) <i>W</i>	Number of kernels in 25 cubic centimeters <i>N</i>
Expansion..... <i>V</i> .....		−0.59±0.022	−0.31±0.031	0.38±0.029
Percentage of soft starch..... <i>S</i> .....	−0.59±0.022		.42±.028	−.47±.026
Weight 100 kernels..... <i>W</i> .....	−.31±.031	.42±.028		−.84±.010
Number of kernels in 25 cubic centimeters..... <i>N</i> .....	.38±.029	−.47±.026	−.84±.010	
Length of kernels..... <i>L</i> .....	−.44±.028	.19±.033	.09±.034	−.12±.033
Breadth of kernels..... <i>B</i> .....	−.29±.031	.15±.033	.16±.033	−.18±.033
Thickness of kernels..... <i>T</i> .....	−.17±.033	.17±.033	.08±.034	−.13±.033

Character	Length of kernels (millimeters) <i>L</i>	Breadth of kernels (millimeters) <i>B</i>	Thickness of kernels (millimeters) <i>T</i>
Expansion..... <i>V</i> .....	−0.44±0.028	−0.29±0.031	−0.17±0.033
Percentage of soft starch..... <i>S</i> .....	.19±.033	.15±.033	.17±.033
Weight 100 kernels..... <i>W</i> .....	.09±.034	.16±.033	.08±.034
Number of kernels in 25 cubic centimeters..... <i>N</i> .....	−.12±.033	−.18±.033	−.13±.033
Length of kernels..... <i>L</i> .....		.19±.033	.07±.034
Breadth of kernels..... <i>B</i> .....	.19±.033		.09±.034
Thickness of kernels..... <i>T</i> .....	.07±.034	.09±.034	

The correlations between expansion and the first three kernel characters confirm the results of the preliminary study on White Rice pop corn, although the coefficients for weight of kernel and for number of kernels in a given volume are somewhat lower. The correlations involving expansion all show that within this sample those ears with the smaller kernels and those with kernels containing the least proportion of soft starch, in general, give the greatest increase in volume on popping. The percentage of soft starch in the endosperm had the greatest influence on popping yield of any of the kernel characters considered in this study, the coefficient of correlation in this case being −0.59 and 27 times its probable error. The conclusion that ears with the most horny and vitreous kernels tend to give the largest popping yields appears to be fully warranted.

The further analysis of the various relations shows that length, breadth, and thickness of kernel are all negatively correlated with the expansion. This would be expected, as these measurements largely determine the size of the kernel. The correlation of expansion is greatest with length of kernel and least with thickness of kernel. All of these correlation coefficients are statistically significant.

Considering the correlations of kernel characters among themselves, the greatest is between number of kernels per given volume and

weight of kernels and, of course, is negative. Interesting points brought out in Table 4 are the relatively high correlations between weight and starchiness ( $0.42 \pm 0.028$ ) and between size of kernels (as measured by number of kernels in 25 c. c.) and starchiness ( $-0.47 \pm 0.026$ ). These values indicate that large kernels are much more likely than small ones to have a large proportion of soft starch and suggest that one reason for the relatively poor popping quality of the ears with the larger kernels may be the associated higher percentage of soft starch which they contain.

The remaining correlations among the kernel characters are comparatively small. Those coefficients of 0.11 or more are over three times their probable errors and may be considered as probably significant. It is rather surprising that the correlations between weight of kernel and length, breadth, and thickness of kernel are not higher. Length and thickness are more highly correlated with starchiness than with weight, and breadth is correlated about equally with starchiness and with weight.

#### MULTIPLE CORRELATIONS

In the gross correlations considered in the previous paragraphs, the tendencies toward concomitant variation between poppability and certain individual kernel characters have been pointed out. These kernel characters, in turn, have been shown to be more or less highly correlated among themselves. In order to ascertain the relationships between expansion and the combined effect of all the other characters reported, or various groups of them, the method of multiple correlations was used. The coefficients of multiple correlations of expansion with various combinations of kernel characters are given in Table 5.

TABLE 5.—Coefficients of multiple correlation between expansion and various combinations of kernel characters, ranked according to their numerical value

[For explanation of symbols, see Table 3]

Characters	Coefficient (R)	Characters	Coefficient (R)	Characters	Coefficient (R)	Characters	Coefficient (R)
V (SWNLBT).....	0.731	V (SL).....	0.678	V (SW).....	0.597	V (WNB T).....	0.478
V (SWNLB).....	.729	V (SWNBT).....	.674	V (WNLBT).....	.589	V (NB T).....	.458
V (SNLBT).....	.718	V (SWNB).....	.673	V (WNLB).....	.587	V (LT).....	.457
V (SWNL T).....	.715	V (SWN T).....	.644	V (NLBT).....	.587	V (WNB).....	.445
V (SWNL).....	.714	V (SWN).....	.643	V (NLB).....	.578	V (WNT).....	.417
V (SNLB).....	.701	V (SNBT).....	.635	V (WNL T).....	.564	V (WB T).....	.416
V (SWLBT).....	.697	V (SNB).....	.633	V (WNL).....	.561	V (NB).....	.410
V (SLBT).....	.696	V (SWB T).....	.630	V (NL T).....	.557	V (NT).....	.402
V (SWLB).....	.695	V (SB T).....	.629	V (WLB T).....	.553	V (WB).....	.395
V (SLB).....	.694	V (SWB).....	.628	V (NL).....	.548	V (WN).....	.355
V (SNLT).....	.688	V (SB).....	.626	V (WLB).....	.541	V (WT).....	.344
V (SNL).....	.686	V (SNT).....	.608	V (WL T).....	.528	V (BT).....	.325
V (SWLT).....	.684	V (SN).....	.604	V (WL).....	.513		
V (SWL).....	.681	V (SWT).....	.601	V (LB T).....	.499		
V (SLT).....	.681	V (ST).....	.597	V (LB).....	.482		

When all six kernel characters are considered with expansion,  $R=0.731$ . This value for  $R$  may be substituted in the formula  $100 (1 - \sqrt{1 - R^2})$  to find the proportion of variability in expansion directly associated with the kernel characters studied. The value obtained, 31.8 per cent, indicates that approximately one-third of this variability is accounted for by variations having to do with size

and starchiness of the unpopped kernels. Other factors, such, perhaps, as the size of the starch grains or the tenacity or elasticity of the surrounding matrix, may play an important part in determining the degree of expansion.

In the sample studied, however, fairly reliable predictions of poppability could be made on the basis of the size and soft-starch content of the kernels, two easily observable characters.

The importance of the influence of starchiness on popping quality may be noted from the fact that the correlation between expansion and percentage of soft starch ( $r_{vs} = -0.593$ , Table 4) is slightly greater than the multiple correlation between expansion and all the kernel characters studied other than starchiness ( $R_v(\text{WNLBT}) = 0.589$ , Table 5).

#### PARTIAL CORRELATIONS

The partial correlation method is especially valuable for purposes of analysis in a study of this kind, in which several related characters are more or less highly correlated among themselves.

The various partial correlations resulting from the study are given in Table 6. In each case the first two letters indicate the variables for which the correlation is calculated with the characters indicated by the subsequent letters held constant. The coefficients, therefore, measure the correlation between expansion and one of the kernel characters, the influence of various combinations of the other characters being eliminated. The coefficients for any two characters are arranged in order of their magnitude.

The partial correlation coefficients for expansion and percentage of soft starch are high throughout, irrespective of what combinations of other characters are held constant. Of all the kernel characters studied, the proportion of soft starch seems to be most closely associated with popping expansion and to be most nearly free from influence by the other characters studied.

The partial correlations of expansion with weight of kernel present an interesting situation. The coefficients are all negative when the number of kernels is variable, but all positive in those combinations in which number of kernels is held constant. In other words, the popping expansion is highest in general in the lightest kernels, but in the case of kernels of a given size the popping expansion is slightly greater in the heavier ones. This suggests at once that probably there is a positive relation between popping expansion and specific gravity. The fact that pop corn has a considerably higher test weight per measured bushel than does field corn lends support to this hypothesis. No determinations of kernel density were made on the ears studied.

The partial coefficients for expansion with number of kernels per 25 cubic centimeters are all positive. There is a decided drop in those coefficients, however, in which starchiness is held constant. The smaller coefficients in every instance are combinations which contain starchiness as one of the constant factors.

The correlations of expansion with length of kernel are negative and are all of undoubted significance, the smallest coefficient being thirteen times its probable error. These coefficients differ little, irrespective of what characters are held constant.

TABLE 6.—*Partial correlations of expansion with each kernel character studied when various combinations of the other characters are held constant*

[For explanation of symbols see Table 3]

Characters	r	Characters	r	Characters	r	Characters	r
VS. T.	-0.58	VW. NT.	0.01	VL. T.	-0.43	VB. WL.	-0.20
VS. B.	-.58	VW. NLBT.	.01	VL. W.	-.43	VB. SL.	-.20
VS. L.	-.58	VW. NL.	.01	VL. WT.	-.43	VB. SLT.	-.20
VS. LB.	-.57	VW. NBT.	.02	VL. N.	-.42	VB. WLT.	-.19
VS. BT.	-.57	VW. N.	.02	VL. WN.	-.42	VB. SWL.	-.19
VS. LT.	-.57	VW. NLB.	.02	VL. NT.	-.42	VB. WNL.	-.19
VS. LBT.	-.56	VW. NB.	.03	VL. WNT.	-.42	VB. NL.	-.19
VS. W.	-.54	VW. NSLT.	.04	VL. SW.	-.41	VB. SWLT.	-.19
VS. WB.	-.53	VW. NSL.	.05	VL. S.	-.41	VB. SWNL.	-.19
VS. WT.	-.52	VW. NST.	.05	VL. SN.	-.41	VB. WNL T.	-.19
VS. WL.	-.52	VW. NSLBT.	.05	VL. SWT.	-.41	VB. NL T.	-.19
VS. WBT.	-.52	VW. NSLB.	.05	VL. SWN.	-.41	VB. SWNL T.	-.19
VS. WLB.	-.52	VW. NS.	.05	VL. ST.	-.41	VB. SNLT.	-.18
VS. WLT.	-.51	VW. NSBT.	.05	VL. SNT.	-.41	VB. SNLT.	-.18
VS. WLB T.	-.51	VW. NSB.	.06	VL. SWNT.	-.41	VB. L.	-.16
VS. NW.	-.51	VN. L.	.37	VL. B.	-.40	VB. W.	-.16
VS. N.	-.51	VN. T.	.37	VL. WB.	-.40	VB. B.	-.15
VS. NWB.	-.50	VN. LT.	.36	VL. BT.	-.40	VB. LB.	-.15
VS. NB.	-.50	VN. B.	.35	VL. WBT.	-.40	VB. WL.	-.14
VS. NWT.	-.50	VN. LB.	.35	VL. NB.	-.40	VB. WB.	-.14
VS. NT.	-.50	VN. BT.	.34	VL. WNB.	-.40	VB. WLB.	-.13
VS. NWBT.	-.50	VN. LBT.	.34	VL. NBT.	-.40	VB. N.	-.13
VS. NWL.	-.50	VN. W.	.24	VL. WNB T.	-.40	VB. WN.	-.13
VS. NBT.	-.50	VN. WL.	.22	VL. SWB.	-.39	VB. NL.	-.12
VS. NWLB.	-.49	VN. WB.	.22	VL. SWNB.	-.38	VB. WNL.	-.12
VS. NL.	-.49	VN. WT.	.22	VL. SNT.	-.38	VB. NB.	-.12
VS. NLB.	-.49	VN. WLB.	.22	VL. SWBT.	-.38	VB. WNB.	-.12
VS. NWLT.	-.49	VN. WLT.	.21	VL. SB.	-.38	VB. NLB.	-.11
VS. NWLB T.	-.49	VN. WBT.	.21	VL. SWNB T.	-.38	VB. WNLB.	-.11
VS. NLT.	-.49	VN. WLB T.	.20	VL. SBT.	-.38	VB. S.	-.09
VS. NLBT.	-.49	VN. S.	.15	VB. T.	-.28	VB. SW.	-.09
VW. T.	-.30	VN. SL.	.14	VB. W.	-.26	VB. SL.	-.08
VW. L.	-.30	VN. ST.	.14	VB. S.	-.25	VB. SN.	-.08
VW. LT.	-.30	VN. SLT.	.14	VB. WT.	-.25	VB. SWL.	-.08
VW. B.	-.28	VN. SW.	.13	VB. WN.	-.25	VB. SWN.	-.08
VW. LB.	-.28	VN. SWL.	.12	VB. ST.	-.25	VB. SWB.	-.08
VW. BT.	-.27	VN. SWT.	.12	VB. N.	-.25	VB. SB.	-.08
VW. LBT.	-.27	VN. SLB.	.12	VB. SW.	-.24	VB. SNL.	-.07
VW. SL.	-.09	VN. SLBT.	.12	VB. WNT.	-.24	VB. SWLB.	-.07
VW. SLT.	-.09	VN. SB.	.12	VB. NT.	-.24	VB. SLB.	-.07
VW. S.	-.09	VN. SWB.	.12	VB. SWT.	-.24	VB. SWNL.	-.07
VW. ST.	-.09	VN. SWLT.	.12	VB. SWN.	-.24	VB. SNT.	-.07
VW. SLBT.	-.07	VN. SBT.	.12	VB. SN.	-.24	VB. SWLB.	-.07
VW. SLB.	-.07	VN. SWLB.	.12	VB. SWNT.	-.23	VB. SNLB.	-.07
VW. SBT.	-.06	VN. SWBT.	.11	VB. SNT.	-.23	VB. SWNLB.	-.06
VW. SB.	-.06	VN. SWLB T.	.11	VB. L.	-.23		
VW. NLT.	-.01	VL. SNB.	-.43	VB. LT.	-.22		

Correlations of 0.11 are more than three times their probable errors, those of 0.14 more than four times, those of 0.17 more than five times, those of 0.31 more than ten times, those of 0.43 more than fifteen times, and correlations of more than 0.51 are more than twenty times their probable errors.

The correlations between expansion and breadth of kernel and between expansion and thickness of kernel are all small, particularly those of the latter group.

#### DISCUSSION

Of the characters studied, the proportion of soft starch seems to be the outstanding determining influence. Sectioning kernels from each individual ear is rather tedious, and it requires experience to estimate with accuracy the relative amounts of soft starch. The high correlation between starchiness and size of kernel, however, may serve as a rough guide to eliminate many of the ears having the softest kernels. Growers frequently have a tendency to select large seed ears, which usually have kernels above the average in size and starchiness and may represent a more or less distant contamination with field corn. This practice should be avoided if quality is to be maintained.

The fact that the results of the two experiments here reported, made with samples from different varieties, confirm each other so closely lends support to the view that the relationships are of a general nature and are not limited to the varieties here considered. It should not be overlooked, however, that although some of the correlations are high the multiple correlation between expansion and all the kernel characters combined was only 0.731. The associations between kernel characters and popping expansion should be a valuable aid in selection of pop corn for seed, but they can not entirely replace a popping test of prospective seed ears as a means of crop improvement.

#### SUMMARY

In pop corn a moisture content of about 12 per cent is optimum for greatest expansion in popping.

Within a given variety, those ears bearing kernels with the least soft starch are likely, on the average, to give the largest expansion in popping.

Similarly, those ears having smaller kernels are more likely to give a higher expansion on popping than those with larger kernels.

Study of these kernel characters should be a valuable aid in selecting seed to maintain and improve the popping quality of a strain of pop corn, although it can not wholly supplant an actual popping test of individual ears.

# THE RELATIVE ASSIMILATION BY DAIRY COWS OF CLOVER AND ALFALFA HAYS AND OF RATIONS OF DIFFERENT CALCIUM AND PHOSPHORUS CONTENT<sup>1</sup>

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## INTRODUCTION

The rôle of the Ca/P ratio in the diet as a cause of rickets or osteoporosis in rats has been the subject of considerable investigation. Park (6)<sup>2</sup> has reviewed the literature bearing on this point. It has been shown that excessive amounts of either calcium or phosphorus in the ration will produce rickets. When both are present in about equal and sufficient quantities and the other components of the ration are satisfactory, rickets does not develop.

It has seemed likely that an unbalancing of the Ca/P ratio in the rations of dairy cows might lead to poor assimilation of calcium and phosphorus and a condition of osteoporosis, as indicated by negative balances. In the usual feeding practice no thought is given to the relative amounts of calcium and phosphorus in the feeds. Although the calcium and phosphorus contents of feeds vary somewhat, it is possible that some attention given to this point may result in improved animal nutrition. It was with the idea of investigating this possibility that the present work was undertaken, it being thought that the investigation of the assimilability of the calcium and phosphorus of alfalfa and clover hays would be both interesting and profitable.

## COWS USED

The experiments were conducted in an attempt to learn whether there is a difference in the degree of assimilability by dairy cows of (1) the calcium and phosphorus of clover and alfalfa hays and (2) the calcium and phosphorus in diets in which the ratio Ca/P is varied. Three cows were used for the work. Cow 253, a purebred Holstein, was used in both experiments. The other cows, 412 and 441, were purebred Jerseys. Cow 412 was to have been used throughout both experiments; but since much difficulty was experienced in inducing her to consume her ration, she was replaced in the second experiment by cow 441. The cows were approximately 7 years old and had just finished a year's official test for milk production about four months previous to the beginning of the experiment. In the interim all the cows had calved and were therefore at their highest plane of milk production. They had had no pasture or green feed for more than a year before the experiment was begun.

<sup>1</sup> Received for publication July 14, 1927; issued November, 1927.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 635.

## FEEDS USED

During the first experiment the cows received grain mixture 75, consisting of 40 kgm. wheat bran, 40 kgm. corn meal, 20 kgm. linseed-oil meal, and 1 kgm. salt. In addition to the grain, they received as roughage during the first three weeks a well-cured clover hay grown on the dairy experiment farm at Beltsville, Md. The clover was cut May 26, 1925, in the early stage of maturity and was fed in March, 1926. During the latter half of the first experiment alfalfa hay was substituted for clover. This alfalfa hay was a baled product received from Kansas City on March 30, 1925, and fed in April, 1926. It had therefore been at the farm for more than a year before it was used, and as it was received at Beltsville in early spring it was probably cut six to nine months before it was shipped. It appeared to be of good quality, but nothing is known regarding its curing. Both hays were chopped and sacked before use.

During the second experiment the cows received grain mixture 160, a feed of low phosphorus content, consisting of 10 kgm. corn meal, 20 kgm. corn gluten feed, 40 kgm. linseed-oil meal, 30 kgm. ground oats, and 1 kgm. salt. The same lot of alfalfa hay used in the first experiment was fed throughout the second experiment. During the last three weeks of the second experiment sodium phosphate was added to the grain in sufficient amount to obtain a value of about 1.25 for the Ca/P ratio in the ration. Monosodium phosphate was fed May 26 to 31 and June 4 to 8, and disodium phosphate was fed the remainder of the time.

## EXPERIMENTAL METHODS

With the exception of the phosphorus determination, the methods of analysis used in these experiments were the same as those employed in previous work in this laboratory (3, 4, 5). Instead of the tedious gravimetric method for phosphorus used in previous experiments at Beltsville, the colorimetric method, as recently modified by Roe, Irish, and Boyd (7), was employed. However when a pure sodium phosphate solution was used as a standard for comparison, it was not found possible to obtain correct results by this method, since the different concentrations of salts and acids in the various materials analyzed apparently affected the depth of color obtained. For each particular type of material to be analyzed, therefore (feces, milk, grain, or hay), a solution of a sample of that material on which a gravimetric determination had already been made, was used as the standard for colorimetric comparison of the other samples of the same type. This was found to give very satisfactory results and to effect a considerable saving of time.

The details of the experimental routine were much the same as those described in a former publication of this bureau (5). The feces and urine were collected automatically. The samples were preserved in the same manner as formerly. The feeding and the collection periods were computed in the same manner; that is, the feeding periods preceded by two days the collection periods in order to compensate for the lag in elimination of excreta. Tap water was furnished the cows by automatic drinking cups, which were filled from individual storage tanks over each stall. The daily intake of water was

recorded, the average of which varied from 47 liters in March for cow 412 to 80 liters in June for cow 253. As 1 liter of tap water was found to contain only 2 mgm. of calcium the effect of the water on the total calcium intake was regarded as insignificant. Throughout the experiments the cows were confined in a barn without exposure to sunlight. A self-recording thermometer gave information in regard to temperature, the variation of which was from 44° to 85° F. during the experiments. The daily rations of hay and grain were given about 2 p. m. and 5 a. m.

### EXPERIMENTAL DATA

The percentage composition of the feeds used throughout the experiments is shown in Table 1. The amounts of feeds consumed are given in Table 2 and the percentage composition of the milk in Table 3.

TABLE 1.—Composition of feeds as fed in both experiments

Period	Feed	H <sub>2</sub> O	Ca	P	N
		<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Mar 17-Apr 6	Clover hay	12.70	1.384	0.153	1.903
Apr. 7-May 4	Alfalfa hay (I)	12.04	1.532	.172	2.654
May 5-June 13	do.	12.86	1.518	.171	2.629
June 14-15	Alfalfa hay (II)	9.93	1.275	.237	2.721
Mar. 17-Apr 6	Grain 75 (I)	12.71	.0995	.745	2.574
Apr. 7-22	do.	12.23	.100	.749	2.588
Apr. 23-May 4	Grain 75 (II)	12.93	.0805	.535	2.273
May 5-25	Grain 160	9.74	.308	.524	3.812
May 26-June 15	do.	10.12	.306	.522	3.796

TABLE 2.—Feed records of the cows used in both experiments

Week	Cow 253			Cow 412		
	Grain offered	Hay offered	Feed refused	Grain offered	Hay offered	Feed refused
	<i>Kgm.</i>	<i>Kgm.</i>	<i>Kgm.</i>	<i>Kgm.</i>	<i>Kgm.</i>	<i>Kgm.</i>
Mar 17-23	56.0	42.0	0.5 hay	55.9	39	8.16 hay and grain.
Mar. 24-30	60.2	46.2	0.4 hay	45.5	34	0.8 hay.
Mar. 31-Apr 6	63.0	49.0	3.6 hay and grain	53.2	35	0.6 hay.
Apr. 7-13	57.6	53.2	None	49.4	30	None.
Apr. 14-20	60.2	53.2	do.	51.2	35	Do.
Apr. 21-27	59.2	53.2	do.	34.2	35	Do.
Apr 28-May 4	60.2	53.2	do.	41.4	35	Do.
				Cow 441		
May 5-11	55.9	53.2	None	56	54.5	None.
May 12-18	60.2	53.2	do.	56	56	Do.
May 19-25	60.2	56.0	do.	56	56	Do.
May 26-June 1	56.0	56.0	do.	56	56	Do.
June 2-8	56.0	56.0	do.	56	55	Do.
June 9-15	56.0	56.0	do.	56	56	Do.

TABLE 3.—Average daily composition of milk from cows used in both experiments

Week	Cow 253			Cow 412		
	Ca	P	N	Ca	P	N
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Mar. 19-25.....	0.105	0.091	0.475	0.145	0.119	0.603
Mar. 26-Apr. 1.....	.104	.089	.454	.146	.119	.601
Apr. 2-8.....	.106	.088	.435	.141	.119	.602
Apr. 9-15.....	.101	.091	.468	.140	.114	.584
Apr. 16-22.....	.101	.090	.457	.142	.117	.620
Apr. 23-29.....	.100	.092	.457	.138	.115	.595
Apr. 30-May 6.....	.098	.093	.476	.141	.118	.606
				Cow 441		
May 7-13.....	.093	.092	.474	0.148	0.123	0.657
May 14-20.....	.096	.091	.481	.147	.124	.666
May 21-27.....	.096	.091	.485	.145	.120	.662
May 28-June 3.....	.097	.092	.484	.145	.119	.666
June 4-10.....	.097	.095	.481	.148	.123	.691
June 11-17.....	.097	.093	.483	.145	.122	.684

On March 12, 1926, cows 253 and 412 were placed in metabolism stalls. Cow 253 weighed 513 kgm. at the beginning of the experiment and 507 kgm. at the end. Cow 412 weighed 481 kgm. at the beginning of the experiment and 426 kgm. at the end. The experiment terminated on May 6. The records of balances and assimilations are presented in Tables 4 and 5.

The second experiment followed immediately. In anticipation of replacing cow 412 by cow 441, the latter was placed in a metabolism stall on May 5, and both cow 253 and cow 441 were given the ration which they were to receive in the second experiment. Cow 253 weighed 507 kgm. at the beginning of the experiment and 501 kgm. at the end. Cow 441 weighed 518 kgm. at the beginning of the experiment and 517 kgm. at the end. The experiment terminated on June 17. The results of the experiment are given in Tables 6 and 7.

TABLE 4.—Daily calcium, phosphorus, and nitrogen balances and assimilations and milk yield of cow 253, March 19 to May 6, 1926

## CLOVER PERIOD

Week	Ca in urine and feces	Ca in milk	Total Ca ex-creted	Ca intake	Ca balance	Ca assimilation		Milk yield	P in urine and feces	P in milk	Total P ex-creted	P intake	P balance	P assimilation		N in urine and feces	N in milk	Total N ex-creted	N intake	N balance	N assimilation	
						Gm.	Per cent of intake							Gm.	Per cent of intake						Gm.	Per cent of intake
Mar. 19-25	82.5	19.5	102.0	90.9	-11.1	8.4	9.2	18.6	53.0	10.9	71.9	68.8	-3.1	13.8	20.0	213.4	88.3	301.7	318.7	+17.0	105.3	33.0
Mar. 26-Apr. 1	91.4	20.0	111.4	98.8	-11.6	8.4	8.4	18.2	60.3	17.1	77.4	74.2	-3.2	12.8	18.6	248.1	87.3	335.4	345.9	+10.5	97.8	28.3
Apr. 2-8	98.9	20.5	119.4	103.8	-15.6	4.9	4.7	19.4	59.8	17.0	70.8	73.3	-1.6	15.4	20.5	263.2	84.2	347.4	354.3	+6.9	91.1	25.7
Average	90.9	20.0	110.9	98.2	-12.8	7.2	7.4	19.1	58.4	17.0	75.4	72.8	-2.7	14.3	19.7	241.6	86.6	328.2	339.6	+11.5	98.1	29.0

## ALFALFA PERIOD

Week	Ca in urine and feces	Ca in milk	Total Ca ex-creted	Ca intake	Ca balance	Ca assimilation		Milk yield	P in urine and feces	P in milk	Total P ex-creted	P intake	P balance	P assimilation		N in urine and feces	N in milk	Total N ex-creted	N intake	N balance	N assimilation	
						Gm.	Per cent of intake							Gm.	Per cent of intake						Gm.	Per cent of intake
Apr. 9-15	108.5	20.5	129.0	124.7	-4.3	16.1	12.9	20.3	62.7	18.4	81.1	74.7	-6.4	12.0	16.6	306.9	94.9	401.8	414.7	+12.9	107.8	26.0
Apr. 16-22	110.0	20.0	130.0	125.0	-5.0	15.1	12.1	19.8	60.5	17.9	78.4	77.5	-0.9	17.0	21.9	318.8	90.7	409.5	424.3	+14.7	105.4	24.9
Apr. 23-29	111.6	20.3	131.9	123.7	-8.2	12.1	9.8	20.3	44.8	18.7	63.5	63.6	+0.1	18.8	20.6	290.7	92.9	383.6	401.7	+18.1	111.0	27.6
Apr. 30-May 6	110.5	19.7	130.2	123.4	-6.8	12.9	10.4	20.1	42.7	18.7	61.4	59.1	-2.4	16.3	27.7	306.4	95.7	402.1	397.2	-4.9	90.8	22.9
Average	110.2	20.1	130.3	124.2	-6.1	14.1	11.3	20.1	52.7	18.4	71.1	68.7	-2.4	16.0	24.0	305.7	93.6	399.3	409.5	+10.2	103.8	25.4

TABLE 5.—Daily calcium, phosphorus, and nitrogen balances and assimilations and milk yield of cow 412, March 19 to May 6, 1926

## CLOVER PERIOD

Week	Ca in urine and feces	Ca in milk	Total Ca excreted	Ca intake	Ca balance	Ca assimilation		Milk yield	P in urine and feces	P in milk	Total P excreted	P intake	P balance	P assimilation		N in urine and feces	N in milk	Total N excreted	N intake	N balance	N assimilation
						Gm.	Per cent of intake							Gm.	Per cent of intake						
Mar. 19-25	67.4	17.2	84.8	75.8	-9.0	8.5	11.0	12.1	49.1	14.4	63.5	61.1	-2.4	12.0	19.2	205.5	72.8	278.2	278.2	-0.1	72.7
Apr. 1-8	67.0	17.8	84.8	76.6	-8.2	9.6	12.5	12.6	48.9	15.0	63.9	64.3	+0.4	15.4	24.0	219.7	76.0	295.7	295.7	-6.8	24.0
Apr. 2-8	67.1	17.2	84.3	73.5	-14.8	2.5	3.4	11.8	50.6	14.0	61.9	55.8	-8.8	5.3	9.4	204.0	70.9	274.9	288.9	-17.4	20.8
Apr. 2-8	64.1	17.2	81.3	77.4	-3.9	13.3	17.1	11.8	47.8	14.1	61.9	63.1	+1.2	15.3	24.2	192.7	71.4	264.1	288.0	+23.9	96.3
Average	67.4	17.4	84.8	75.8	-9.0	8.5	11.0	12.1	49.1	14.4	63.5	61.1	-2.4	12.0	19.2	205.5	72.8	278.2	278.2	-0.1	72.7

## ALFALFA PERIOD

Week	Ca in urine and feces	Ca in milk	Total Ca excreted	Ca intake	Ca balance	Ca assimilation		Milk yield	P in urine and feces	P in milk	Total P excreted	P intake	P balance	P assimilation		N in urine and feces	N in milk	Total N excreted	N intake	N balance	N assimilation
						Gm.	Per cent of intake							Gm.	Per cent of intake						
Apr. 9-15	67.7	15.4	83.1	72.7	-10.4	5.0	6.9	11.0	53.1	12.5	65.6	60.2	-5.4	7.1	11.8	232.3	64.2	296.5	296.4	-0.1	64.1
Apr. 16-22	62.4	16.6	79.0	83.9	+4.9	21.5	25.6	11.7	43.0	13.7	56.7	63.4	+6.7	20.3	32.1	230.6	72.5	303.1	322.0	+18.9	91.3
Apr. 23-29	70.8	14.3	85.1	81.0	-4.1	10.2	12.6	10.4	29.6	11.9	41.5	39.4	-2.1	9.8	24.9	181.2	61.6	242.8	250.6	+7.7	69.3
Apr. 30-May 6	70.3	15.6	85.9	81.4	-4.5	11.0	13.6	11.0	26.5	13.0	39.5	40.2	+0.7	13.7	34.1	191.3	66.8	258.1	267.1	+9.0	73.8
Average	67.8	15.5	83.3	79.8	-3.5	11.9	14.7	11.0	38.1	12.8	50.8	50.8	0.0	12.7	25.7	208.9	66.3	275.1	284.0	+8.9	75.1

TABLE 6.—Daily calcium, phosphorus, and nitrogen balances, assimilations, milk yield, and Ca/P intake of cow 253, May 7 to June 17, 1926

## LOW PHOSPHORUS PERIOD

Week	Ca in urine and feces	Ca in milk	Total Ca excreted	Ca intake	Ca balance	Ca assimilation		Milk yield	P in urine and feces	P in milk	Total P excreted	P intake	P balance	P assimilation		N in urine and feces	N in milk	Total N excreted	N intake	N balance	N assimilation
						Gm.	Per cent of intake							Gm.	Pr. ct. of intake						
May 7-13	126.2	18.2	144.4	140.0	-4.4	13.7	9.8	2.55	19.5	43.6	63.1	54.8	-6.8	11.2	20.5	376.4	92.6	469.0	504.2	+35.2	127.8
May 14-20	123.0	18.5	141.5	141.9	+0.4	18.9	13.3	2.44	19.3	42.6	60.2	58.1	-2.1	15.5	26.7	387.2	92.8	480.0	527.6	+47.6	140.4
May 21-27	126.5	18.3	147.8	147.9	+0.1	18.4	12.5	2.52	19.1	51.0	68.4	58.7	-9.6	7.8	13.2	404.9	92.7	497.6	538.1	+40.6	133.3
Average	126.2	18.3	144.6	143.3	-1.3	17.0	11.9	2.50	19.3	45.7	63.4	57.2	-6.2	11.5	20.1	389.5	92.7	482.2	523.3	+41.1	133.8

## HIGH PHOSPHORUS PERIOD

May 28-June 3	116.2	18.4	134.6	145.9	+11.3	20.7	20.4	1.10	19.0	99.2	116.7	132.9	+16.2	33.7	25.4	269.5	91.9	401.4	514.0	+52.6	144.5
June 4-10	120.7	18.1	138.8	145.9	+7.1	25.2	17.3	1.39	18.7	82.1	109.9	104.6	-4.7	22.5	21.5	384.3	80.9	471.3	514.0	+39.7	120.6
June 11-17	120.4	17.7	138.1	140.4	+2.2	20.0	14.2	1.28	18.3	90.8	107.8	109.6	+1.8	16.7	17.1	394.8	88.2	483.0	516.1	+33.1	121.3
Average	119.1	18.1	137.2	144.1	+6.9	25.0	17.3	1.26	18.7	90.7	108.1	115.7	+7.6	25.0	21.3	382.9	90.0	472.9	514.7	+41.8	131.8

TABLE 7.—Daily calcium, phosphorus, and nitrogen balances, assimilations, milk yield, and Ca/P intake of cow 441, May 7 to June 17, 1926

## LOW PHOSPHORUS PERIOD

Week	Ca in urine and feces	Ca in milk	Total Ca excreted	Ca intake	Ca balance	Ca assimilation		Milk yield	P in urine and feces	P in milk	Total P excreted	P intake	P balance	P assimilation		N in urine and feces	N in milk	Total N excreted	N intake	N balance	N assimilation	
	Gm.	Gm.	Gm.	Gm.	Gm.	Per cent of intake	Gm.	Kg. m	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	P. ct. of intake	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	Per cent of intake
May 7-13	117.3	19.3	136.6	142.8	+6.2	25.5	17.9	2.59	40.1	16.1	58.2	55.2	+3.0	15.2	27.5	373.6	85.9	459.5	509.6	+50.2	136.1	28.7
May 14-20	124.3	19.2	143.5	146.1	+2.6	21.7	14.9	2.63	39.1	16.2	55.3	55.6	+0.3	16.5	29.7	388.1	86.9	475.0	515.3	+40.3	127.2	24.7
May 21-27	132.5	18.1	150.6	146.1	-4.5	13.6	9.3	2.63	55.0	15.0	70.0	55.6	-14.4	.6	1.1	405.9	82.7	488.6	515.3	+26.7	103.4	21.2
Average	124.7	18.9	143.6	145.0	+1.4	20.3	14.0	2.62	44.7	15.8	60.5	55.5	-5.0	10.8	19.4	389.2	85.2	474.4	513.4	+39.1	124.2	24.2

## HIGH PHOSPHORUS PERIOD

May 28-June 3	124.0	17.8	141.8	145.9	+4.2	21.9	15.0	1.10	104.4	14.6	119.0	132.9	132.9	+13.9	28.4	21.4	401.8	81.6	483.4	514.9	+30.6	112.2	21.8
June 4-10	123.6	17.5	141.1	143.8	+2.6	20.1	14.0	1.38	81.8	14.6	96.4	104.4	104.4	+8.0	22.6	21.7	403.7	81.9	485.6	510.2	+24.6	106.5	20.9
June 11-17	124.2	17.0	146.2	140.4	-5.8	11.2	8.0	1.28	92.8	14.3	107.1	108.6	108.6	+2.5	16.7	15.3	409.8	80.0	489.8	518.1	+26.3	106.3	20.6
Average	125.6	17.4	143.0	143.4	+0.3	17.7	12.3	1.25	93.0	14.5	107.5	115.6	115.6	+8.1	22.6	19.5	405.1	81.2	486.3	513.4	+27.2	108.3	21.1

\* Estimated from data for 6 days only.

By using a method adopted in a former paper (5), the body losses and gains of calcium and phosphorus for each cow are shown graphically in Figure 1. Each cow is assumed to have 7,000 gm. of calcium and 3,255 gm. of phosphorus in her bones at the beginning of the experiment. The record of cow 253 is shown as a continuous graph throughout both experiments, whereas the graphs for the other animals are shown only for the experiment in which they were used. It is apparent that in the beginning of the clover and alfalfa experiment both cows showed a fairly rapid loss of both calcium and phosphorus, followed by a slower steady loss. In the low and high phosphorus

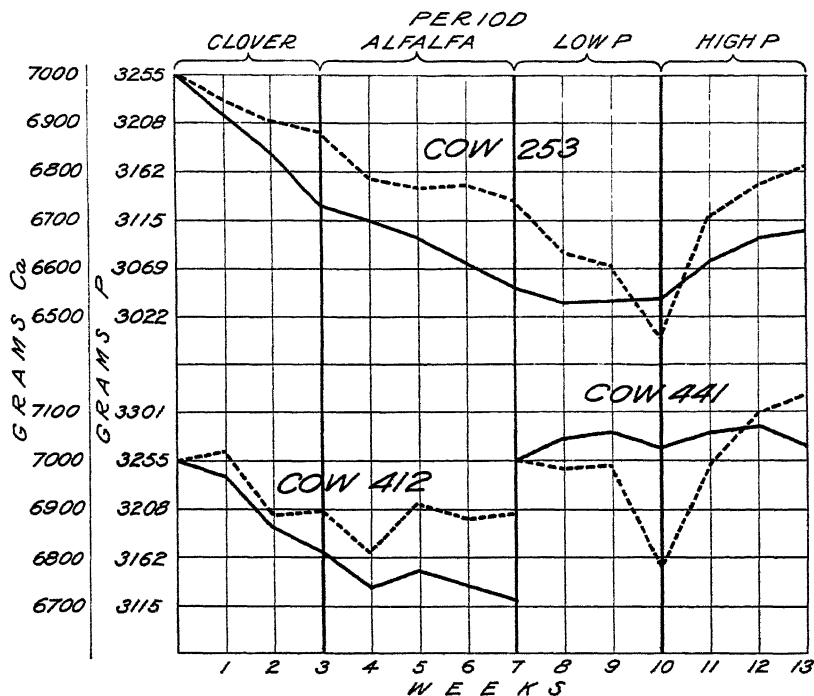


FIG. 1.—Curves showing the losses and gains of calcium and phosphorus by cows during the experiments. The solid line represents the change in the calcium content in the body; the broken line the change in the phosphorus content

experiment the results were quite different. The losses in the beginning were followed by gains, and at the end of the experiment both animals had more calcium and phosphorus in their bodies than at the beginning.

#### DISCUSSION

Cow 253 shows negative calcium and phosphorus balances through practically all the first experiment. The negativity of the calcium balance is reduced more than one-half, however, by substituting alfalfa for clover. The assimilation also shows a very decided improvement in the alfalfa period. The differences in the phosphorus balances and assimilations are not so marked, although they show a tendency in the same direction. These improved balances and

assimilations are accompanied by an increased milk yield. The nitrogen balances show that the cow suffered no digestive disturbance. Cow 412 likewise showed negative calcium and phosphorus balances through the greater part of the experiment. There was a general improvement in the character of her balances and assimilations in the alfalfa period, although her milk yield dropped somewhat. Considerable difficulty was encountered in inducing her to consume an adequate ration. The character of her nitrogen balances, three of which were negative, shows a somewhat disturbed digestive condition. Both cows assimilated a greater proportion of calcium during the alfalfa period in spite of the fact that the calcium intake was greater.

It would appear from this experiment that calcium is better assimilated by dairy cows in the form of alfalfa than in the form of clover. Apparently both hays were of good quality although the alfalfa when fed was probably a year older than the clover. It is not known what factor facilitates the assimilation of calcium from alfalfa. Whether it is the fat-soluble vitamin or some other unsuspected factor is a matter of speculation. In a recent publication from this laboratory it was shown that alfalfa contains citric, malic, and malonic acids (8). Possibly the presence of these acids exerts a favorable effect on the assimilability of calcium. The expressed juice of green alfalfa has a  $P_H$  of 5.28.<sup>3</sup> It has been reported that a rickets-producing diet can be changed into a nonrickets-producing diet by increasing the acidity (6).

In the second experiment, where the phosphorus content of the ration was varied, cow 253, except during one week, showed positive calcium balances throughout. Calcium balances and assimilations were decidedly better in the high phosphorus period. The phosphorus balances were negative in the low phosphorus period and positive in the high phosphorus period. The phosphorus assimilation was larger also in the high phosphorus period and the percentage assimilation, even with a doubled phosphorus intake, remained constant. The nitrogen record indicates that the cow's digestion was normal. The performance of cow 441 was less noteworthy. Her calcium balances and assimilations were slightly poorer on the high phosphorus ration. Her phosphorus balances and assimilations were better, however, in the high phosphorus period, and the percentage assimilations, with a doubled phosphorus intake, remained constant. The nitrogen record shows that her digestion was normal.

Apparently cows can assimilate calcium and phosphorus when the Ca/P ratio in the feed is as high as 2.5, but a more favorable value for this ratio is probably from 1.0 to 1.5.

It is worthy of note that in this second experiment positive calcium and phosphorus balances have been obtained with a cow receiving a ration of alfalfa hay and a good grain mixture and yielding 19 kgm. of milk daily. So far as the authors know, such a performance has not previously been noted for a cow on so simple a ration. Hart (1) found that a cow could be kept in positive calcium balance while on a ration of good alfalfa hay, corn silage, and grain, and yielding about 19 kgm. of milk. He (1, 2) also succeeded in obtaining positive calcium balances with cows on rations containing green feed. But the

<sup>3</sup> Unpublished data obtained at this laboratory.

great majority of calcium balances which have been determined in cows giving large amounts of milk on winter feeds have been negative.

The writers' observations on balance experiments lead them to believe that three conditions are important for insuring equilibrium or a positive balance in dairy cows in respect to calcium and phosphorus.

1. A sufficient quantity of calcium and phosphorus must be supplied in the ration.

2. The calcium and phosphorus should be supplied in about equal quantities. The amount of calcium probably should not exceed the amount of phosphorus by more than 50 per cent.

3. The calcium should be supplied as a constituent of natural feeds in a ration including a good quality of legume hay, preferably alfalfa, and a good grain mixture.

#### SUMMARY

Metabolism experiments with two lactating cows show a better assimilation of calcium and phosphorus from alfalfa hay than from clover hay.

Metabolism experiments involving the use of rations where the content of phosphorus was increased by the addition of sodium phosphate suggest a better assimilation of calcium and phosphorus when the value of the Ca/P ratio of the feed is 1.25 than when it is 2.5.

Positive calcium and phosphorus balances have been observed in the case of a cow on a ration of good alfalfa hay and a good grain mixture and yielding 19 kgm. of milk.

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# THE EFFECT OF INANITION UPON THE YIELD AND COMPOSITION OF COWS' MILK<sup>1</sup>

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## INTRODUCTION

Very little work has been reported on the effect of inanition upon the yield and composition of milk. Only one record was found in the literature of an inanition period as long as 48 hours. Several investigators report upon the effects of underfeeding. Lami (2)<sup>2</sup> starved a cow for 36 hours and at the end of the starvation period found increases in the percentages of total solids (13.6 to 14.3 per cent) and in nitrogenous matter and ash (4.2 to 6.25 per cent.) He found a slight reduction in fat (4.4 to 4.15 per cent) and a decided reduction in lactose (5 to 3.9 per cent).

Eckles and Palmer (1) studied the influence of underfeeding upon the milk of a number of cows. They found that high fat tests uniformly accompanied underfeeding, that there was no uniform effect on the percentages of the other milk components, and that the percentage of fat was not affected in any of the experiments.

Porcher (4) states that starvation for two days showed no appreciable injury to the milk.

Ragsdale and Swett (5) showed that a sudden reduction of the total amount of feed to one-half the normal amount increased the percentage of fat and decreased the amount of milk produced.

Taylor and Husband (6), in their study of the effect of variations in the nature of the diet upon the percentage composition of the milk of the goat, state that (1) the percentages of protein, fat, and ash vary inversely and the percentage of lactose varies directly as the daily volume, the greatest variation being shown by the fat and the least by the inorganic elements; (2) there is an inverse relationship between the percentage of lactose and the percentages of all the other constituents of the milk, this being particularly apparent in the case of the fat. They suggest that the quantity of lactose elaborated by the mammary glands controls the daily volume of the milk, and that, therefore, the rate of its elaboration controls the rate of milk secretion.

The present study was undertaken to learn the effect of total absence of feed upon the yield and the composition of the milk produced.

## DESCRIPTION OF ANIMALS USED

Three cows, Nos. 1, 11, and 742, were used in this investigation. These cows, while in the experimental herd of the dairy department at the University of Illinois, gave a positive reaction to the tuberculin test and were isolated from the herd until slaughtered.

<sup>1</sup> Received for publication May 31, 1927; issued November, 1927. Presented before the division of biological chemistry at the seventy-third meeting of the American Chemical Society, Richmond, Va., Apr. 11 to 16, 1927.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 644.

Cow No. 1, born October 4, 1923, was predominantly Holstein, probably less than one-quarter Guernsey. She calved March 10, 1926, and was in her first lactation during this investigation. She was bred May 27, 1926.

Cow No. 11, born May 12, 1921, was a high-grade Holstein. She calved December 11, 1925, and was in her third lactation. She was due to calve February 27, 1927. Cows Nos. 1 and 11 were condemned for slaughter August 11, 1926, following a positive reaction to the tuberculin test. The latest previous test, January 26, 1926, had given negative results.

Cow No. 742, born December 10, 1922, was a second generation Guernsey-Holstein cross. She calved January 13, 1927, for her second lactation. She was condemned February 15, 1927, following a positive reaction to the tuberculin test. The latest previous test, December 15, 1926, was negative.

The cows were in good flesh at the beginning of the investigation and were apparently in good physical condition. Post-mortem examinations by competent veterinary clinicians showed very slight glandular lesions and that tuberculosis was present only in its early stage.

#### EXPERIMENTAL PROCEDURE

The cows were given their customary feed and care during a 10-day preliminary period and were milked regularly twice each day at about 6 a. m. and 6 p. m. During this period three composite samples of milk were taken for each cow. The first sample represented all the milk produced during the first four days. The second and third samples each represented all the milk produced in successive three-day periods. Following the preliminary period the cows were given water but no feed. The inanition period continued five days for cow No. 11 and six days for cows Nos. 1 and 742. The treatment of the animals, except for the absence of feed, was precisely the same as during the preliminary period.

TABLE 1.—Composition of milk from cow No. 1 preceding and during the inanition period

Date (1926)	Milk yield	Total solids	Fat	Protein	Lactose (gravity)	Ash	Specific gravity	Total solids	Fat	Protein	Lactose	Ash
	Lbs.	P. ct.	P. ct.	P. ct.	P. ct.	P. ct.		Lbs.	Lbs.	Lbs.	Lbs.	Lbs.
Oct. 25 a.....	<sup>b</sup> 13.4	12.63	4.03	3.27	4.40	0.706	1.0342	1.6924	0.5400	0.4382	0.5896	0.09460
Oct. 28 a.....	<sup>d</sup> 13.3	12.94	4.22	3.24	Lost.	.708	1.0356	1.7210	.5613	.4309	Lost.	.09416
Oct. 31 a.....	<sup>d</sup> 13.5	12.94	4.14	3.32	4.47	.710	1.0297	1.7409	.5589	.4482	.6035	.09666
Oct. 31, p. m....	13.1	12.72	4.02	3.30	4.35	.712	1.0305	1.6663	.5295	.4323	.5999	.09327
Nov. 1, a. m....	10.6	12.64	4.02	3.24	4.30	.738	1.0293	1.3398	.4201	.3434	.4558	.07823
Nov. 1, p. m....	4.4	16.28	7.53	3.85	3.80	.803	1.0277	.7163	.3312	.1694	.1672	.03533
Nov. 2, a. m....	3.3	18.04	8.70	3.50	3.10	.930	1.0283	.5953	.2871	.1155	.1023	.03069
Nov. 2, p. m....	2.0	23.12	13.34	4.97	2.60	1.063	1.0290	.4624	.2668	.0994	.0520	.02126
Nov. 3, a. m....	1.5	23.79	13.94	5.62	2.04	1.197	1.0286	.3569	.2091	.0843	.0306	.01796
Nov. 3, p. m....	.7	25.41	15.17	5.72	1.60	1.207	1.0271	.1779	.1062	.0400	.0112	.00845
Nov. 4, a. m....	.9	26.14	14.36	6.80	1.50	1.115	1.0286	.2353	.1292	.0612	.0135	.01004
Nov. 4, p. m....	.7	24.18	13.77	6.23	1.77	1.112	1.0271	.1693	.0964	.0436	.0124	.00778
Nov. 5, a. m....	.7	26.22	14.91	6.41	1.83	1.114	1.0250	.1835	.1044	.0449	.0128	.00780
Nov. 5, p. m....	.5	28.83	18.23	6.96	1.55	1.097	1.0275	.1442	.0912	.0348	.0078	.00548
Nov. 6, a. m....	.9	30.17	19.15	7.09	1.70	1.078	.0265	.2715	.1724	.0638	.0153	.00970

<sup>a</sup> A composite sample made by taking proportional amounts from the milk produced at each milking for 4 days.

<sup>b</sup> Average milk yield per milking for 4 days.

<sup>c</sup> Composite samples made by taking proportional amounts from the milk produced at each milking for 3 days.

<sup>d</sup> Average milk yield per milking for 3 days.

All samples were analyzed for percentages of total solids, fat, total protein, lactose, and ash. The methods of analysis employed by the Association of Official Agricultural Chemists were used, except that about 5 gm. of sample were employed for the fat determination by the Roese-Gottlieb method, and about 10 gm. of sample and 3 c. c. of strong nitric acid were used in the ash determination. The lactose was determined by the reduction method, the cuprous oxide being weighed directly. The specific gravities were taken at 20° C. with a chainomatic specific gravity balance.

The results obtained, including milk yields, percentages of total solids, fat, protein, lactose, and ash, and the yields of the components of the milk, are given in Tables 1, 2, and 3, and are graphically represented in Figures 1, 2, 3, and 4.

TABLE 2.—*Composition of milk from cow No. 11 preceding and during the inanition period*

Date (1926)	Milk yield	Total solids	Fat	Protein	Lactose (gravity)	Ash	Specific gravity	Total solids	Fat	Protein	Lactose	Ash
	<i>Lbs.</i>	<i>P. ct.</i>	<i>P. ct.</i>	<i>P. ct.</i>	<i>P. ct.</i>	<i>P. ct.</i>		<i>Lbs.</i>	<i>Lbs.</i>	<i>Lbs.</i>	<i>Lbs.</i>	<i>Lbs.</i>
Oct. 25 a-----	<sup>b</sup> 12.9	12.83	3.93	3.48	4.45	0.748	1.0351	1.6551	0.5070	0.4489	0.5741	0.09649
Oct. 28 a-----	<sup>d</sup> 13.3	12.86	3.46	3.41	Lost.	.731	1.0348	1.7104	.4602	.4535	Lost.	.09722
Oct. 31 c-----	<sup>d</sup> 13.9	12.90	3.84	3.49	4.56	.740	1.0301	1.7931	.5338	.4851	.6338	.10286
Oct. 31, p. m.---	13.3	12.70	3.73	3.53	4.50	.729	1.0331	1.6891	.4988	.4695	.5985	.09696
Nov. 1, a. m.---	11.8	13.04	4.00	3.47	4.45	.698	1.0298	1.5387	.4720	.4045	.5251	.08236
Nov. 1, p. m.---	6.7	14.49	5.28	3.50	4.15	.756	1.0311	.9708	.3538	.2345	.2781	.05065
Nov. 2, a. m.---	4.8	16.92	7.22	4.30	3.68	.893	1.0290	.8122	.3466	.2064	.1766	.04286
Nov. 2, p. m.---	3.1	21.08	10.09	5.77	2.82	1.129	1.0341	.6535	.3128	.1789	.0874	.03500
Nov. 3, a. m.---	2.6	21.98	10.67	6.14	2.77	1.220	1.0351	.5715	.2774	.1596	.0720	.03172
Nov. 3, p. m.---	2.5	21.38	10.07	6.37	2.62	1.177	1.0335	.5345	.2518	.1593	.0655	.02942
Nov. 4, a. m.---	2.2	23.49	11.80	6.59	2.62	1.086	1.0306	.5168	.2596	.1450	.0576	.02389
Nov. 4, p. m.---	2.3	23.90	12.38	6.80	2.70	1.274	1.0342	.5497	.2847	.1564	.0621	.02930
Nov. 5, a. m.---	1.6	26.48	14.88	6.88	2.55	1.225	1.0294	.4237	.2381	.1101	.0408	.01960

<sup>a</sup> A composite sample made by taking proportional amounts from the milk produced at each milking for 4 days.

<sup>b</sup> Average milk yield per milking for 4 days.

<sup>c</sup> Composite samples made by taking proportional amounts from the milk produced at each milking for 3 days.

<sup>d</sup> Average milk yield per milking for 2 days.

TABLE 3.—*Composition of milk from cow No. 742 preceding and during inanition period*

Date (1927)	Milk yield	Total solids	Fat	Protein	Lactose (gravity)	Ash	Specific gravity	Total solids	Fat	Protein	Lactose	Ash
	<i>Lbs.</i>	<i>Pct.</i>	<i>Pct.</i>	<i>Pct.</i>	<i>Pct.</i>	<i>Pct.</i>		<i>Lbs.</i>	<i>Lbs.</i>	<i>Lbs.</i>	<i>Lbs.</i>	<i>Lbs.</i>
Feb. 21 a-----	<sup>b</sup> 20.5	14.19	5.27	3.06	4.64	0.717	1.0307	2.9090	1.0804	0.6273	0.9512	0.14698
Mar. 1 c-----	<sup>d</sup> 21.8	13.16	4.28	3.00	4.87	.688	1.0302	2.8689	.9330	.6540	1.0617	.14998
Mar. 4 c-----	<sup>d</sup> 22.6	12.96	4.18	2.93	4.80	.673	1.0298	2.9290	.9447	.6622	1.0948	.15210
Mar. 4 p. m.---	22.1	12.74	4.15	2.84	4.76	.642	1.0300	2.8155	.9172	.6276	1.0520	.14138
Mar. 5 a. m.---	22.9	13.22	4.69	2.73	4.55	.649	1.0299	3.0274	1.0740	.6252	1.0420	.14862
Mar. 5 p. m.---	12.6	17.54	9.23	2.99	4.60	.726	1.0244	2.2100	1.1630	.3767	.5796	.09148
Mar. 6 a. m.---	9.3	20.00	11.57	3.40	3.73	.804	1.0251	1.8600	1.0760	.3162	.3460	.07477
Mar. 6 p. m.---	8.0	18.22	9.56	3.18	3.80	.790	1.0247	1.4576	.7648	.2544	.3010	.06320
Mar. 7 a. m.---	7.6	18.22	9.72	3.30	3.78	.782	1.0219	1.3847	.7387	.2508	.2873	.05943
Mar. 7 p. m.---	7.1	17.32	8.74	3.35	3.72	.828	1.0274	1.2297	.6205	.2378	.2641	.05879
Mar. 8 a. m.---	4.8	19.17	10.28	3.53	3.66	.906	1.0245	.9202	.4994	.1694	.1757	.04349
Mar. 8 p. m.---	7.2	16.66	8.32	2.93	3.83	.762	1.0271	1.1995	.5980	.2146	.2758	.05486
Mar. 9 a. m.---	9.1	17.74	9.69	2.75	3.93	.676	1.0243	1.16143	.8818	.2502	.3576	.06152
Mar. 9 p. m.---	7.4	18.44	10.11	3.04	3.79	.781	1.0226	1.3646	.7811	.2250	.2805	.05779
Mar. 10 a. m.---	.5	19.06	10.18	3.34	4.07	.829	1.0214	.0953	.0509	.0167	.0204	.00414

<sup>a</sup> A composite sample made by taking proportional amounts from the milk produced at each milking for 4 days.

<sup>b</sup> Average milk yield per milking for 4 days.

<sup>c</sup> Composite samples made by taking proportional amounts from the milk produced at each milking for 3 days.

<sup>d</sup> Average milk yield per milking for 3 days.

## RESULTS

The milk yield and the content of the various components showed only the normal fluctuations during the preliminary feeding period. However, after the beginning of the inanition period, the yield of milk dropped very rapidly and marked changes in the composition were found. Cow No. 1 produced an average of 13.4 pounds of milk per milking during the preliminary period. After three days without feed her production had dropped to less than 1 pound per milking and varied from 0.5 to 0.9 of a pound each milking during

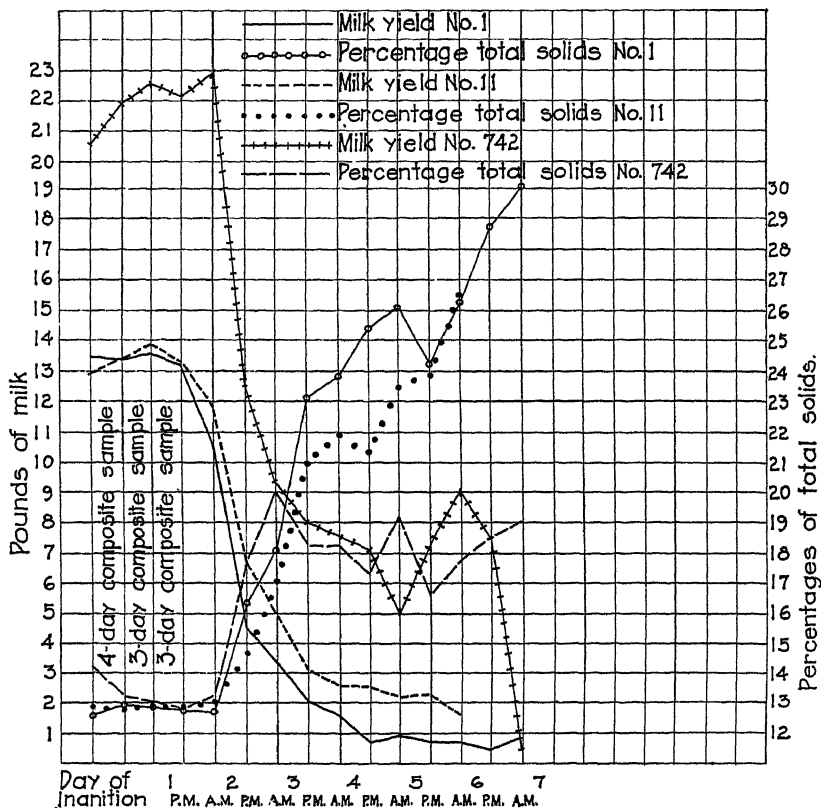


FIG. 1.—Milk yields and percentages of solids preceding and during inanition period

the remainder of the period. The percentage composition of her milk also changed very radically. The total solids increased from an average of 12.82 per cent during the preliminary period to 30.17 per cent for the last milking during inanition. The fat increased from an average of 4.12 to 19.15 per cent; the protein from an average of 3.28 to 7.09 per cent; the ash from an average of 0.710 to 1.207 per cent in the seventh milking during inanition. In the remaining five milkings the ash varied between 1.078 and 1.115 per cent. The lactose averaged 4.43 per cent for seven days of the preliminary period (this determination on the second composite sample was lost) and decreased to 1.6 per cent in the seventh milking of the inanition period. It varied from 1.50 to 1.83 per cent in the remaining five milkings.

The trend of the production and of the composition of the milk was the same for cow No. 11 as for cow No. 1. The milk yield decreased from an average of 13.3 pounds per milking during the preliminary period to 2.6 pounds at the end of three days' inanition, and decreased slowly through the remaining four milkings. The percentage of total solids increased from an average of 12.86 to 26.48 per cent; fat from 3.76 to 14.88 per cent; protein from 3.46 to 6.88 per cent; and ash from 0.740 to 1.274 per cent. The lactose

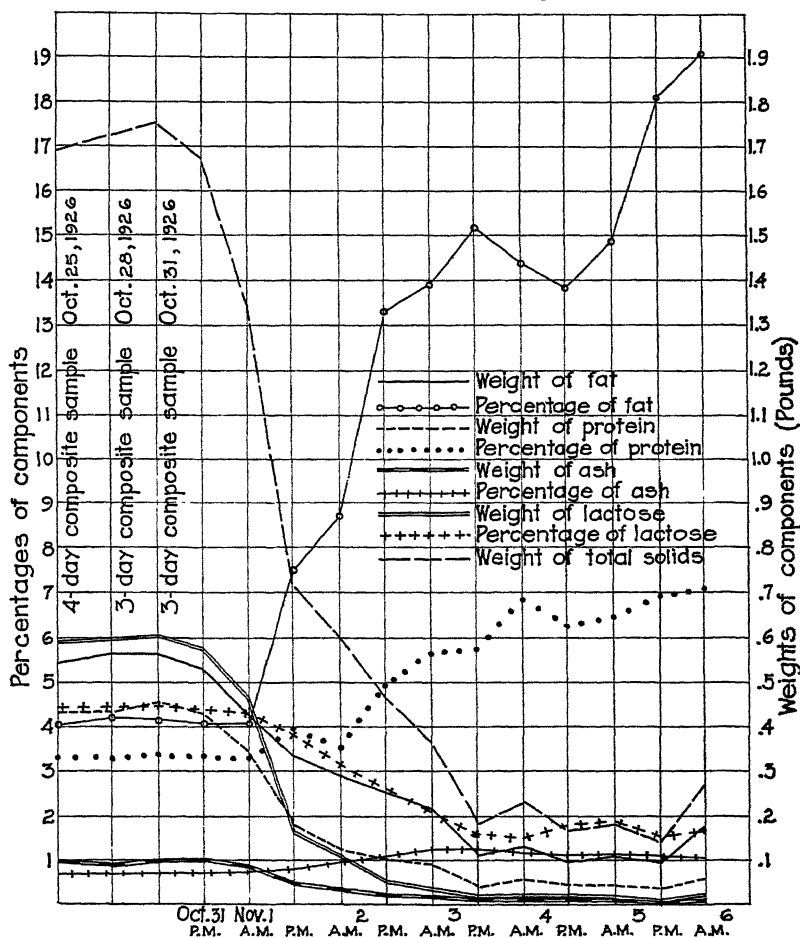


FIG. 2.—Percentages and yields of milk components for cow No. 1, preceding and during inanition period

averaged 4.50 per cent during 7 days of the preliminary 10-day period, and decreased to 2.55 per cent during inanition.

The analyses of the milk samples from cow No. 742 showed much less variation than for the other two cows. The milk yield decreased from an average of 21.5 pounds per milking during the preliminary period to 8 pounds at the end of three days' inanition, and fluctuated between 4.8 and 9.1 pounds during the next six milkings. During the last 12 hours of inanition only 0.5 pound of milk was produced, while during the preceding 12 hours the production was 7.4 pounds.

The percentage of total solids increased from an average of 13.49 to a maximum of 19.06; and the fat increased from an average of 4.63 to a maximum of 11.57 per cent at the beginning of the third day of inanition and fluctuated between 8.32 and 10.28 per cent during the remainder of the inanition period. The protein averaged 3.01 per cent during the preliminary period and varied between 2.73 and 3.53 per cent during inanition, without showing any regular increase. The lactose decreased from an average of 4.76 per cent in the pre-

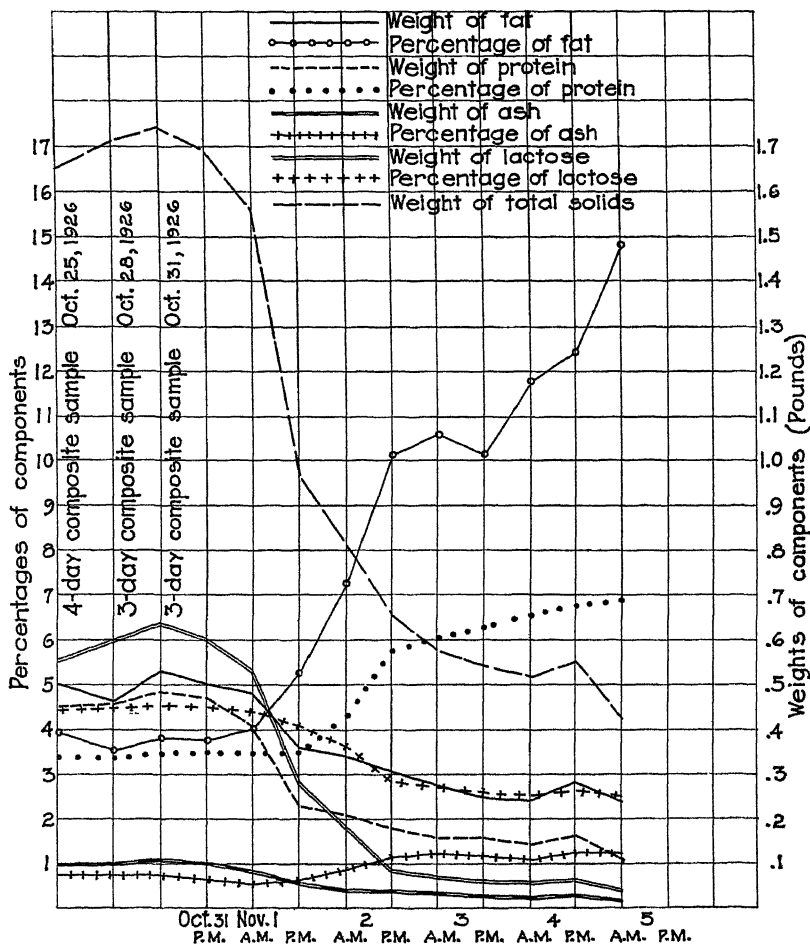


FIG. 3.—Percentages and yields of milk components for cow No. 11, preceding and during inanition period

liminary period to 3.66 per cent for the eighth milking during inanition, then increased slightly, varying from 3.79 to 4.07 per cent during the last four milkings. The value for the last milking (4.07 per cent) is, however, decidedly below the preliminary average for this cow. The ash content averaged 0.696 per cent during the preliminary period and for the most part was above this value during inanition, reaching for one milking a maximum of 0.906 per cent.

The specific gravity of the milk tended to be lower during inanition than during the normal feeding period.

# DISCUSSION

The results obtained in this investigation show that inanition exerts a profound influence upon the yield and the composition of the milk of the dairy cow. As the milk production decreased the percentage content of all the components except lactose increased. The increases in percentages of fat were greatest for cow No. 1 and showed a maximum of 4.65 times the average during the preliminary 10-day feeding period. For cows Nos. 11 and 742, the increases were, respectively, 3.95 and 2.50 times the preliminary 10-day aver-

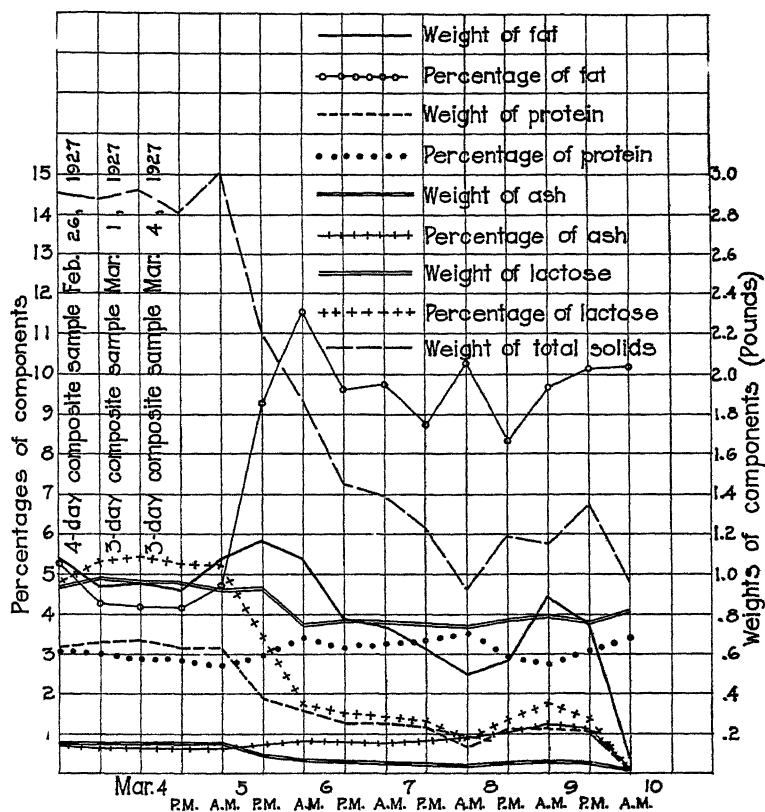


FIG. 4.—Percentages and yields of milk components for cow No. 742, preceding and during inanition period

age. The maximum percentage of total solids for cow No. 1 was 2.35 times the preliminary average. For cows Nos. 11 and 742, it was, respectively, 2.06 and 1.49 times the preliminary average. The maximum percentages of protein for the three cows were, respectively, 2.14, 1.99, and 1.17 times the preliminary average. The maximum percentages of ash were, respectively, 1.70, 1.72, and 1.30 times the preliminary average.

The lactose, however, decreased from an average of 4.43 per cent to a minimum which was 33.9 per cent of that value for cow No. 1. The corresponding values for cows Nos. 11 and 742, were, respectively, 56.7 and 76.9 per cent of the preliminary average.

These changes in composition of milk, which were caused by lack of feed, agree with the observations of other investigators cited in the introduction to this paper. The reduction in the percentage of lactose also supports the statement made from this laboratory that the lactose content of milk is especially sensitive to physiological disturbances (3). As the quantity of lactose produced by the cows studied in this investigation decreased much more rapidly than did the volume of milk, it appears that the suggestion of Taylor and Husband (6) does not apply to cows subjected to inanition, since they state that the quantity of lactose elaborated by the mammary glands controls the daily volume of the milk, and that, therefore, the rate of its elaboration controls the rate of milk secretion.

The reduction in the milk flow was less rapid and the variations in composition were less marked for cow No. 742 than for the other two cows. This may be explained by the fact that this cow had begun her lactation only a short time previous to the beginning of the investigation, while the other two cows were approaching the end of their lactations. This explanation is in accord with the belief of Eckles and Palmer (1, p. 69), that the milk flow of the cow is stimulated by two factors chemical and nervous. It is the opinion of these investigators that the chemical factor is more or less independent of the food supply and predominates for a period of time after parturition; and that the nervous factor is almost entirely dependent on the food supply and predominates or replaces the chemical stimulus after the lactation period has attained a certain stage.

#### SUMMARY

The composition of the milk of three cows subjected to inanition has been determined and has been compared with the composition of the milk of the same cows when kept under normal conditions just previous to the inanition period.

It has been shown (1) that as the period of inanition progresses the milk flow decreases, the percentages of total solids, fat, protein, and ash increase, while the specific gravity and the percentage of lactose decrease, and (2) that the changes are not so marked when inanition occurs early in the lactation period.

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# PARADICHLOROBENZENE AS AN ANTHELMINTIC<sup>1</sup>

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## INTRODUCTION

Paradichlorobenzene has in recent years become well known as an insecticide. It has been found to be very effective as a moth exterminator (3),<sup>2</sup> and has been advocated as a valuable aid in controlling the peach tree borer. Some information is also available concerning its efficacy against external parasites of animals (1).

The chemical and physical properties of paradichlorobenzene are given by Duckett (3, p. 6), by Konantz (8), and by Sollmann (10). Concerning its physiological properties, Duckett (3, p. 7), quoting from Francis and Fortescue-Brickdale (4, p. 99), says that "the benzene halogen derivatives have a slight odor, are insoluble in water, volatilize without decomposition, and are very stable. \* \* \* Corresponding to their stability it is found that the halogen is not split off in the organism, and that they do not show hypnotic properties. \* \* \* Chlorobenzene acts on the spinal cord to a greater extent than benzene."

According to Sollmann (10) nothing is known of the pharmacology of paradichlorobenzene. Duckett (3, p. 1) quotes Curschman as saying that poisoning of human beings by paradichlorobenzene through contact with the skin is impossible and that inhalation of the vapors of this product is perfectly harmless; and, furthermore, that paradichlorobenzene is harmful to human beings only in cases of internal application of large quantities, say from 30 to 40 grains.

Sollmann (10) reports that dogs tolerated doses of paradichlorobenzene up to 15 gm. without showing any ill effects, but he does not consider the drug very effective as an anthelmintic.

Experiments at the Porto Rico Agricultural Experiment Station were undertaken to determine the anthelmintic value of paradichlorobenzene, especially against the hook-worm.

## EXPERIMENTAL DATA

Paradichlorobenzene was administered to dogs (1) as crystals in gelatin capsules in doses of 0.1, 0.3, 0.5, and 1 gm. per kilo of body weight; (2) dissolved in liquid petrolatum or in olive oil, in doses of 1 gm. per kilo of body weight; (3) as crystals in gelatin capsules in doses of 0.5 and 1 gm. per kilo of body weight, followed an hour later by an ounce of liquid petrolatum or of castor oil; and (4) as crystals in gelatin capsules followed immediately by (a) a meal of fat, (b) a meal of whole milk, (c) a meal of lean meat, and (d) a meal of dry bread.

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<sup>1</sup> Received for publication Apr. 6, 1927; issued November, 1927.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 649.

Liquid petrolatum and olive oil were selected as carriers because, the one being a hydrocarbon and the other a bland oil, any anthelmintic action produced would be considered as due to the paradichlorobenzene dissolved in them, since they of themselves are without any such action (6).

The animals were fasted overnight previous to administering the drug, and given no food for a period of two hours following dosing. The feces were then collected for a period of from 48 to 72 hours and examined. Table 1 shows the efficacy of paradichlorobenzene in experiments 1, 2, and 3.

From the table it will be seen that in the case of dog No. 26, in which the worms (*Ancylostoma caninum*) found in the large intestine were considered impotent, paradichlorobenzene, administered in the form of crystals at the rate of 0.1 gm. per kilo of body weight, was inefficacious for roundworms, tapeworms, and whipworms, and 28.5 per cent efficacious against hookworms.

Administered at the rate of 0.3 gm. per kilo of body weight, the drug was inefficacious for roundworms and whipworms, and averaged 2 per cent efficacious against hookworms, and 1.5 per cent against tapeworms, in the case of dogs Nos. 19 and 20.

Administered at the rate of 0.5 gm. per kilo of body weight, the drug was inefficacious for tapeworms, and averaged 25.45 per cent efficacious against hookworms, in the case of dogs Nos. 1, 2, and 19.

Administered at the rate of 1 gm. per kilo of body weight, the drug averaged 66.5 per cent efficacious against roundworms in two trials, and 49.5 per cent against hookworms in the case of dogs Nos. 4, 5, 9, 10, 11, 12, 15, and 16.

Administered dissolved in liquid petrolatum at the rate of 1 gm. per kilo of body weight, the drug was inefficacious for tapeworms and 4.5 per cent efficacious against hookworms, as shown in the case of dog No. 13.

Administered in olive oil at the rate of 1 gm. per kilo of body weight, the drug was 17 per cent efficacious against hookworms in the case of dog No. 14.

Administered in crystal form in gelatin capsules at the rate of 0.5 gm. per kilo of body weight, and followed an hour later by an ounce of liquid petrolatum, the drug was inefficacious for roundworms and tapeworms, and averaged 60.5 per cent efficacious against hookworms and 12.5 per cent efficacious against whipworms, in the case of dogs Nos. 22 and 24.

Administered in crystal form in gelatin capsules at the rate of 1 gm. per kilo of body weight, and followed an hour later by an ounce of liquid petrolatum, the drug was 4.5 per cent efficacious against hookworms and 100 per cent efficacious against whipworms in the case of dog No. 29.

Administered in the form of crystals in gelatin capsules at the rate of 0.5 gm. per kilo of body weight, followed an hour later by an ounce of castor oil, the drug was inefficacious for roundworms, tapeworms, and whipworms, and averaged 15 per cent efficacious against hookworms, as shown in the case of dogs Nos. 23, 25, and 27.

Administered in the form of the crystals in gelatin capsules at the rate of 1 gm. per kilo of body weight, followed an hour later by an ounce of castor oil, the drug was 62.5 per cent efficacious against hookworms in the case of dog No. 28.

TABLE 1.—Efficacy of paradichlorobenzene, administered in several ways, as an anthelmintic for dogs

Dog No.	Weight of dogs	Dose per kilo of body weight	Total dose	Quantity of oil	Ascarids				Hookworms				Tapeworms				Whipworms									
					Number recovered ante mortem	Number recovered post mortem		Percentage of efficacy	Number recovered ante mortem	Stomach	Number recovered post mortem		Percentage of efficacy	Number recovered ante mortem	Stomach	Number recovered post mortem		Percentage of efficacy	Number recovered ante mortem	Stomach	Number recovered post mortem		Percentage of efficacy			
26	2.5	Gm. 0.1	Gm. 0.25	Gm. 0			3		0	6		15		28.50			56		0							
19	3.0	" 0.3	" 0.75	" 0			11		0			43		4.00			1	2	3							
20	4.0	" 0.3	" 1.2	" 0								1,598		41.00					0							
2	5.0	" 0.5	" 2.5	" 0					(b)	950	1	130	10	30.35			11		0							
1	4.0	" 0.5	" 2	" 0					(b)	60	1	4	37	1	5.00			25		0						
17	6.0	" 0.5	" 3	" 0						41					100.00			2		0						
4	5.0	" 1.0	" 5	" 0						28		3			90.00					0						
5	6.0	" 1.0	" 6	" 0											75.00											
9	4.0	" 1.0	" 4	" 0				2	100			9	26													
10	3.5	" 1.0	" 3.5	" 0						102		10		91.00												
11	7.0	" 1.0	" 7	" 0						69		189	12	24.25			1		0							
12	2.0	" 1.0	" 2	" 0	1		4	1	33	6		231		2.60					0							
15	4.5	" 1.0	" 4.5	" 0								19					0		0							
16	6.0	" 1.0	" 6	" 0						4				12.50					0							
13	4.0	" 1.0	" 4	" 15					(b)	5	4	357	12	4.50			10		0							
14	4.0	" 1.0	" 4	" 15					(b)	6		183	31	17.00					0							
22	3.0	" 0.5	" 1.5	" 30			3		0	37		0		100.00			1		(b)							
24	3.5	" 0.5	" 1.75	" 30						54		302	51	21.00					(b)							
29	3.0	" 1.0	" 3	" 30					(b)	5	2	106		4.50					0							
23	3.5	" 1.0	" 3.5	" 30			3		0			11														
25	4.0	" 1.5	" 6	" 30						66		817	298	30.00			1		0							
27	8.0	" 1.5	" 12	" 30						7	1	42	1	15.00												
28	5.0	" 1.0	" 5	" 30					(b)	5		3		62.50					(b)							

\* Administered as crystals in gelatin capsules.

\* Dissolved in liquid petrolatum.

\* Dissolved in olive oil.

\* Gelatin capsules followed in an hour by liquid petrolatum.

\* Gelatin capsules followed in an hour by castor oil.

## TOXICITY

Dogs Nos. 9 and 10 died showing pronounced symptoms of intoxication. The practice of the experiment station has been to fast the animals overnight before administering paradichlorobenzene, and to withhold feed for a period of two hours following dosing. In the case of dogs Nos. 9 and 10, however, the attendant inadvertently gave the animals fat meat immediately after dosing.

The dogs received the drug at about 9.30 a. m., and were down and trembling violently at 1 p. m. Dog No. 9 soon became unconscious and died about 36 hours later without having regained consciousness. Dog No. 10 was not so violently affected, but showed incoordination of movement after 24 hours, walking with an irregular gait or standing with its legs spread far apart.

To learn whether this result was due to the absorption of paradichlorobenzene, dogs Nos. 18 and 21 were given the drug at the rate of 0.5 gm. per kilo of body weight. Dog No. 18 received a piece of fat immediately following dosing, and dog No. 21 was given all the whole milk it would take.

Both dogs showed decided symptoms of intoxication at 1 p. m., but the symptoms were not nearly so pronounced as in the case of dogs Nos. 9 and 10. The symptoms were still evident at 4 p. m. but disappeared before the next morning.

Four days later the experiments were repeated with the same dogs. The same amount of paradichlorobenzene was given as before, but in addition dog No. 18 received lean meat and dog No. 21 dry bread and water. The drug was administered at 9 a. m., and the first symptoms of intoxication were visible at 1 p. m. These passed off rapidly, however, leaving the animals with no apparent permanent ill effect.

Dog No. 21 passed eight ascarids after receiving the first dose of paradichlorobenzene on October 13, and died a few days after receiving the second dose on October 17. No ascarids were found post-mortem, proving the drug to be 100 per cent efficacious against these intestinal worms in this instance. No percentage of efficacy against hookworms was determined in the case of dog No. 21.

## EFFECT ON EDIBILITY OF MEAT ANIMALS

In connection with the experiments with paradichlorobenzene as an anthelmintic the drug was observed to render unfit for human consumption the meat of animals to which it was administered.

Previous to the arrival of the author at the Porto Rico Experiment Station paradichlorobenzene in crystal form had been administered to pigs. One pig, which had received a total of 14 gm. of the drug during a period of four months, was sold on the hoof, that is, before slaughter, as is the local custom. Those who purchased the meat could not eat it and were refunded their money. The meat smelled and tasted of paradichlorobenzene. The second pig, which had been kept as a check on the first experiment, was given 5 gm. of the drug in one dose and was not slaughtered until two weeks later. The meat of this animal also proved to be unfit for human consumption.

One authority reports tasting paradichlorobenzene in the eggs of hens which were fed corn that had been fumigated with the drug. To test the accuracy of the report the author, about December 27, 1925, fed to some of the hens at the station the ground liver of a dog

to which paradichlorobenzene had been administered. Up to and including January 9, 1926, paradichlorobenzene could be tasted in the yolks of the eggs laid by these hens.

### CONCLUSIONS

Administered in doses of 0.1, 0.3, 0.5, and 1 gm. per kilo of body weight in bland oils and in crystal form, followed by a bland oil in some instances, and by a purgative oil in others, paradichlorobenzene is somewhat efficacious against intestinal worms.

The results obtained were not sufficiently uniform to permit classifying paradichlorobenzene as an effective anthelmintic, and are in accordance with the findings of Sollmann (10).

The results from using paradichlorobenzene dissolved in olive oil and alone are in agreement with the opinion expressed in 1918 by Hall (5) that "olive oil seemed to be decidedly contraindicated in [connection] with anthelmintics."

Hall and Foster (6, p. 432) also point out the fact that liquid petrolatum seriously diminished the efficacy of chenopodium, which seems to be true also in the case of paradichlorobenzene.

The results apparently confirm also the hypotheses advanced by Caius and Mhaskar (2) and Hall and Shillinger (7) concerning the relationship of the chlorine content and the solubility of halogen compounds to their anthelmintic efficacies. Paradichlorobenzene contains only 2 atoms of Cl, is practically insoluble in water, and theoretically therefore should be inferior to such compounds as carbon tetrachloride and the new anthelmintic tetrachlorethylene, which contain a larger number of chlorine atoms and are more soluble in water.

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# CYTOLOGICAL STUDIES ON VIRUS DISEASES OF SOLANACEOUS PLANTS<sup>1</sup>

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## INTRODUCTION

Microscopical examinations of the tissues of mosaic-diseased plants have been made by numerous investigators since the earliest recognition of diseases of the plant virus group. With a few exceptions, these investigations have been confined to the study of a single virus disease upon a single species of host. It has now, however, come to be recognized that there are a number of different virus diseases due to distinct and specific viruses, which frequently have an extended host range, and several of which may be capable of infecting the same host plant. In certain instances, where a single plant species or group of species is susceptible to a number of such diseases, considerable difficulty may be experienced in distinguishing clearly between the latter, though in some cases this may be accomplished by means of a comparative study of the host range, symptomatology, and certain other properties of the viruses concerned. It was believed that a comparative cytological study of such a group of diseases upon an extended host range would be of value in determining whether any microscopical features were present which could be correlated with any specific viruses, thereby providing an additional means of identification. Such a study might also be expected to throw further light on the possible relationships of the different viruses. It was especially borne in mind that various investigators have reported the occurrence of vacuolate, protoplasmic bodies in the tissues of certain mosaic-diseased plants, and that there are those who believe these bodies to be a form of protozoan or other living organism and the casual agent of the disease in question. It is therefore of considerable importance to ascertain to what extent such bodies are associated with virus diseases in general, and a cytological study of the nature described should be of value in this connection. A number of virus diseases were available for study on a variety of solanaceous plants, and the results of a cytological examination of these are presented in this paper, the first section dealing with diseases of a group of solanaceous plants, excluding the potato, and the second with diseases of the potato only, most of which are at present believed to be limited to the potato as host.

## LITERATURE REVIEW

There is considerable literature dealing with the histological and cytological aspects of plant virus diseases. This has been reviewed in detail by various writers, notably Goldstein (8)<sup>3</sup> and Smith (31), and

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<sup>2</sup> The thanks of the writer are due Dr. James Johnson, under whose direction this work was carried out, both for his valuable cooperation in supplying the material for examination and for his interest and advice throughout the course of the investigation. The writer is also indebted to Prof. L. R. Jones for his interest in the work and for his helpful criticism.

<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 669.

in the present paper it is proposed to give only a brief consideration to the more important publications.

Histological modifications occurring in mosaic tissues were first described by Woods (34) in connection with tobacco mosaic. This investigator observed a reduction in length of the palisade cells in the lighter areas of the leaf and a decrease in the amount of intercellular space throughout these regions. Iwanowski (10) later confirmed these observations, describing also certain variations in the width of the lamina in the light and dark areas, together with certain changes in the chloroplasts and nuclei. In the dark green areas the palisade tissue was found to consist to two layers of cells.

Similar modifications have since been reported by different investigators in association with various other mosaic diseases, namely, mosaic of cucumber (4), potato (25), tomato, petunia, henbane, pepper, raspberry, pea, bean, clover (3), pecan (28), sugar cane (2, 24), *Datura stramonium*, pokeweed, and *Aquilegia* sp. (31).

The presence of cell inclusions in mosaic tissues was first reported by Iwanowski (10), who described bodies of three types occurring in the chlorotic areas of mosaic tobacco leaves: (1) Colorless, crystalline plates or layers, resembling some waxy material but of lower refractive index, and showing cross-striations on treatment with acids; (2) protoplasmlike bodies, near, or in close connection with, the nuclei, and whose appearance suggested parasitic amoebae (these bodies were apparently distinct from the cell cytoplasm, and were considered as some reaction product of the cells); (3) granular inclusions, which, on staining, resembled zoogloea, consisting of very minute, short rods, usually occurring in the palisade cells and believed to be bacteria.

Inclusions more or less similar to the amoebalike bodies of Iwanowski have since been described by various workers in association with several different virus diseases of plants. Lyon (20) reports the presence of such bodies in the galls produced in Fiji disease of sugar cane,<sup>4</sup> and these are described more fully by Kunkel (17). Both authors believe the bodies to be living organisms. Kunkel gives a detailed account of somewhat similar bodies present in mosaic corn (15), *Hippeastrum equestre* (16, 18), Chinese cabbage (18), and sugar cane (19). Palm (27) gives a fuller description of the vacuolate bodies associated with tobacco mosaic and describes also certain minute granules in the mosaic tissues which he believes to be the causal organism. McKinney, Eckerson, and Webb report the presence of intracellular bodies, somewhat similar to those of mosaic corn, in rosette and mosaic wheat (23) and in mosaic *Hippeastrum johnsonii* (22). K. M. Smith (32) finds amoebalike bodies in mosaic potato, which he considers to be "some degeneration product of the cell," and Goldstein (9) describes protoplasmic inclusions associated with "mosaic diseased" and "dwarfed" dahlias. Further descriptions of the different cell inclusions occurring in mosaic tobacco are given by Rawlins and Johnson (29) and by Goldstein (7, 8). F. F. Smith (31) reports the presence of protoplasmic bodies in the tissues of mosaic tobacco, petunia, *Datura stramonium*, and pokeweed, and in *Euonymus japonicus* affected with an infectious chlorosis.

Several other types of inclusion have been described as associated with certain mosaic tissues. Sorokin (33) describes the formation of

<sup>4</sup> This may or may not be a virus disease

blisterlike spheres from the chloroplasts of mosaic tomato leaves and the occurrence of minute granular bodies in the tissues. Eckerson (5) reports the presence of small bodies, believed to be flagellates, in mosaic tomato, wheat, *Hippeastrum johnsonii*, dahlia, and squash, and Klebahn (14) finds, in the young phloem cells of *Anemone nemorosa* affected with the disease "Alloiophyllie," minute bodies which he considers to be living organisms.

#### VIRUS DISEASES OF SOLANACEOUS PLANTS, EXCLUDING THE POTATO

##### MATERIAL

The material chosen for microscopical examination was selected from a wide range of solanaceous plants of different genera and species affected with one or more of 11 different virus diseases. These diseases have been fully described by Johnson (11, 13), as regards differential host symptoms and other differential properties of the viruses concerned, under the following names: Tobacco mosaic, cucumber mosaic, speckled tobacco mosaic, mild tobacco mosaic, spot necrosis, ring spot, yellow tobacco mosaic, medium tobacco mosaic, bleaching mosaic, tomato stem necrosis, and petunia mosaic. Since the work was carried out in association with Johnson, there is no doubt about the identity of the diseases studied so far as his proposed classification is concerned, and the descriptions of them need not be repeated here. The more common host plants studied are listed in Table 1.

The method employed in these investigations was to inoculate each virus simultaneously to five plants of each host species under study, the individual plants of each species being of the same age and approximately the same size and vigor. The plants were inoculated when quite young and in a rapidly growing condition. Five similar but uninoculated plants served as a control in each trial. All trials were conducted in one greenhouse run at a temperature of 27° to 32° C. When the symptoms of disease were fully developed, material for microscopical examination was carefully chosen in each case from individual leaves of plants which showed the most intense mottling, chlorosis, or other symptoms of disease. At the same time healthy material for comparison was obtained from the control plants. It has been found by various workers, and has been observed by the writer during the present investigation, that the amount of internal modification or variation in mosaic-diseased plants is in general proportional to the intensity of external symptoms, and is more pronounced and varied in the leaves than in any other organ of the plant. Material was therefore selected which might be expected to show as fully developed internal symptoms of disease as possible for any host species in respect to each individual virus, and material which could be considered in all cases as truly comparative. Certain combinations of host and virus which produced only very faint or no symptoms of mottling were frequently not included in the examination. In some cases it was possible to secure material from only a single set of inoculations, though usually several sets were available for study at different times.

The leaf symptoms produced on the different hosts by different viruses were very varied, and Figure 1 illustrates some of the types of mosaic pattern secured which were characteristic of different viruses on tobacco and which were included in the present examination.

## METHODS

The present examination was conducted largely upon material that had been fixed and stained by the usual methods, though fresh unstained material was also studied from time to time by means of

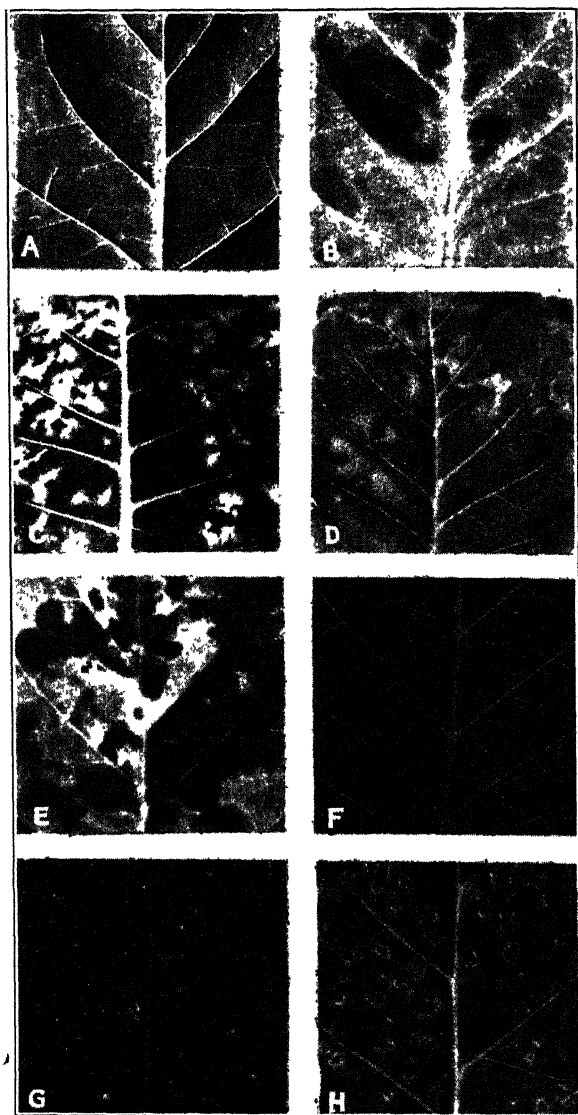


FIG. 1.—Types of mosaic pattern produced on tobacco by different virus diseases: A, Healthy tobacco; B, cucumber mosaic; C, tobacco mosaic; D, tobacco mosaic attenuated by heat; E, yellow tobacco mosaic; F, speckled tobacco mosaic; G, spot necrosis; H, ring spot

free-hand sections to serve as a check upon the other. Small portions, usually square or triangular in shape, with sides measuring a few millimeters, were cut out from the leaf lamina with a sterile safety-

razor blade, placed immediately in the fixing fluid and embedded in the usual way. If the leaves showed any form of mottling, portions were cut out from both the chlorotic and the darker areas and examined separately. Considerable difficulty was experienced in finding a fixing fluid that was satisfactory for all purposes. Many were tested, including Flemming's fluids, chrom-acetic solutions of different strengths, Juel's, Bouin's, and Zenker's fixatives, acetic alcohol, formol alcohol, formol acetic alcohol, Gilson's and other sublimate fixatives and Carnoy's fluid. Several of these gave excellent results with material from young leaves, but proved very unsatisfactory for older tissues. For general purposes formol acetic alcohol (100 parts 50 per cent alcohol, 6.5 parts formalin, and 2.5 parts glacial acetic acid), used for 24 to 48 hours, was considered most suitable and was employed extensively. Carnoy's fluid (6 parts absolute alcohol, 3 parts chloroform, and 1 part glacial acetic acid), used for about 15 minutes, was found to cause less distortion of the cells and was employed for a time, but was subsequently abandoned on account of its rather severe action on the cell contents and of the less satisfactory staining properties which it conferred upon the tissues.

The material was sectioned at a thickness of 7 microns in a plane perpendicular to the leaf surface, and stained with Haidenhain's iron-alum haematoxylin (iron-alum 2 per cent aqueous about 3 hours, haematoxylin  $\frac{1}{2}$  per cent aqueous about 2 hours, destained in iron-alum 1 per cent aqueous). The slides were examined with a 2 mm. apochromatic oil immersion lens and  $7.5\times$  compensating oculars. All photomicrographs were made from stained slides with the 2 mm. lens and a  $6\times$  ocular. All drawings were made with the aid of a camera lucida, using the 2 mm. lens and a  $7.5\times$  ocular.

The amount of material examined for any given virus on any one host varied considerably according to the amount that was available for study and according to certain other considerations. The minimum, however, consisted of four or five leaf portions from the chlorotic areas and about as many from the dark green areas, these being taken from several different leaves and usually from several plants. From each leaf portion, three or more microscopic slides were prepared, 50 or more sections being mounted on each slide. Usually, however, considerably more material than this was examined, and sometimes a great deal was worked through. For example, with petunia mosaic on tobacco, 14 leaf portions from 5 different plants were examined, and with speckled tobacco mosaic on the same host, 15 leaf portions from 4 plants.

#### RESULTS

Certain modifications were observed in the diseased tissues which were of more or less general occurrence throughout the material examined and were not limited to any specific hosts or viruses, though varying according to the intensity of external symptoms developed on the leaves. Similar modifications have already been described by Woods (34), Iwanowski (10) and other investigators, in relation to a number of other mosaic diseases, and these appear to be characteristic of such disease in general. Such modifications relate to changes in the histological structure of the leaf tissues and in the chloroplasts and nuclei.

In the earliest stages of development of the disease, or in other cases where the color modifications occurring in the leaves were only faintly developed, the chloroplasts in the lighter regions appeared somewhat paler and more yellow than those of the normal leaf, indicating some change in the composition of the chlorophyll. In the fixed material this was indicated by a difference in the staining capacity of the chloroplasts, those in the paler areas taking on a somewhat lighter stain. Where the external symptoms of mottling or chlorosis were marked, the chlorophyll had changed from green to yellow, and the plastids were reduced in size, in severe cases of disease sometimes disintegrating and apparently breaking up into small particles. In leaves infected when very young, the chloroplasts did not develop normally, but remained small and fewer in number than in the healthy cell. When placed in water, these plastids often swelled up to form colorless vesicles, as described by Iwanowski (10), and it is thought possible that this may have some bearing on the formation of the "spheres" reported by Sorokin (33). In the dark green areas, on the other hand, the chloroplasts always appeared very large, frequently more numerous, and of a somewhat deeper green than normal.

Modifications in the form of the cells occurred wherever the leaves showed a marked degree of mottling or chlorosis. According to the intensity of such symptoms, the palisade cells in the yellow areas appeared shorter in length and somewhat broader than normal, or reduced to a cuboidal form, while those of the darker areas appeared somewhat longer and thinner than normal and very closely crowded together. In the most severe cases, the palisade tissue of the darker areas consisted of two layers of cells. These modifications resulted in variations in the thickness of the leaf lamina, the lighter areas being reduced in some cases to about two-thirds the thickness of the darker areas.

Similar histological changes and modifications in the chloroplasts were associated with the different potato virus diseases, which will be considered in a later section.

In the more severe forms of disease, the host nuclei were frequently affected, those in the lighter areas of the leaf often appearing shrunken and distorted, and staining more lightly than normal; at other times appearing considerably swollen, with enlarged nucleoli. This latter type was especially characteristic of the potato mosaics. In certain material the nuclei contained square or oblong plates of some dark-staining material, whose nature was not determined, and which, when viewed on end, appeared as narrow rods or bands. Usually one or two of these plates were present in a single nucleus, which contained in addition an apparently unaltered nucleolus; such nuclei were very abundant in some material. They were associated with mild tobacco mosaic on all hosts examined with the exception of *Nicotiana glutinosa*, with bleaching mosaic on pepper and tomato, and with tobacco mosaic on henbane. Their significance is not understood.

It can not, however, be said that any of these modifications occurring in the diseased tissues are sufficiently characteristic of any particular virus or group of viruses to be of value for diagnostic purposes. Their chief significance no doubt lies in the indication which they give that the diseases in question are probably closely related one to another. On the other hand, a study of the association of cell inclusions with the different viruses reveals quite another situation.

In the case of ordinary tobacco mosaic on tobacco (*Nicotiana tabacum*), cell inclusions of two types were found to be invariably present in the chlorotic areas of mottled leaves, namely, the protoplasmic, vacuolate bodies previously described by various writers and which, for convenience, will be referred to as "x-bodies,"<sup>5</sup> and the "striate material" of Rawlins and Johnson (29), which, according to Iwanowski (10), Goldstein (7), and others, results from the action of acids in the fixing fluid on certain waxy-crystalline plates occurring in the living, diseased cells. The appearance of the x-bodies in the stained sections of mosaic tobacco leaves is shown in Plate 1, A, B, C, b. The small, dark-staining bodies described by Rawlins and Johnson (29) were observed only rarely, probably because the material was usually taken during later stages of development of the disease. Contrary to the observations of Rawlins and Johnson (29) and of Smith (31), both x-bodies and striate material appeared to occur as commonly in the field as in the greenhouse. Material taken from 20 different tobacco plants growing in the field, and several feet in height, contained in every case abundant inclusions of both types.

TABLE 1.—Occurrence of cell inclusions in various solanaceous host plants in association with different viruses

Disease	Virus	Hosts										
		<i>Nicotiana tabacum</i>	<i>Nicotiana glauca</i>	<i>Nicotiana glauca</i>	<i>Nicotiana glauca</i>	<i>Nicotiana glauca</i>	<i>Nicotiana glauca</i>	<i>Nicotiana glauca</i>	<i>Nicotiana glauca</i>	<i>Nicotiana glauca</i>	<i>Nicotiana glauca</i>	<i>Nicotiana glauca</i>
Tobacco mosaic.....	Tobacco virus 1.....	++	0	0	++	++	++	++	++	++	++	++
Cucumber mosaic.....	Cucumber virus 1.....	0	0	0	0	0	0	0	0	0	0	0
Speckled tobacco mosaic.....	Tobacco virus 2.....	0	0	0	0	0	0	0	0	0	0	0
Mild tobacco mosaic.....	Tobacco virus 3.....	0	0	0	0	0	0	0	0	0	0	0
Spot necrosis.....	Tobacco virus 4.....	0	0	0	0	0	0	0	0	0	0	0
Ring spot.....	Tobacco virus 5.....	0	0	0	0	0	0	0	0	0	0	0
Yellow tobacco mosaic.....	Tobacco virus 6.....	+	+	+	+	+	+	+	+	+	+	+
Merlino tobacco mosaic.....	Tobacco virus 7.....	0	0	0	0	0	0	0	0	0	0	0
Bleaching mosaic.....	Tobacco virus 8.....	0	0	0	0	0	0	0	0	0	0	0
Tomato stem necrosis.....	Tobacco virus 9.....	0	0	0	0	0	0	0	0	0	0	0

++ indicates that x-bodies and striate material are both present + indicates striate material only present and 0 indicates no inclusions present.

On inoculation of the tobacco-mosaic virus to other solanaceous hosts, these two types of inclusion were again produced in every case where definite mottling or chlorosis of the leaves was secured, and were invariably present in the lighter colored areas, though never occurring in the dark green parts. Table 1 shows a number of host plants examined which were affected with tobacco mosaic, and the inclusions present in each case. In addition to the hosts listed in this table, six others were examined which were affected with tobacco mosaic, and x-bodies and striate material were found to be associated with each. These hosts were: *Physalis alkekengi*, *Solanum cabiliense argenteum*, *S. marginatum*, *S. nigrum*, *S. pyracanthum*, and potato

<sup>5</sup> This term was introduced by Goldstein (7).

(Rural New Yorker and Green Mountain varieties). (Pl. 2, F, and fig. 2, E, J.)

TABLE 2.—Summary of results showing the occurrence of cell inclusions in association with different viruses on all hosts examined

Disease	Virus	Number host species examined	Number showing x-bodies and striate material	Number showing striate material only	Number showing no inclusions
Tobacco mosaic.....	Tobacco virus 1.....	19	16	0	3
Cucumber mosaic.....	Cucumber virus 1.....	11	0	0	11
Speckled tobacco mosaic.....	Tobacco virus 2.....	4	0	0	4
Mild tobacco mosaic.....	Tobacco virus 3.....	7	0	0	7
Spot necrosis.....	Tobacco virus 4.....	9	0	0	9
Ring spot.....	Tobacco virus 5.....	5	0	0	5
Yellow tobacco mosaic.....	Tobacco virus 6.....	9	4	4	1
Medium tobacco mosaic.....	Tobacco virus 7.....	5	1	3	1
Bleaching mosaic.....	Tobacco virus 8.....	7	0	0	7
Tomato stem necrosis.....	Tobacco virus 9.....	7	0	0	7
Petunia mosaic.....	Petunia virus 1.....	1	0	0	1

In all, out of 19 host species examined, 16 showed inclusions of both types. (Table 2.) Of the remaining 3 examined, which contained no inclusions, 1 (*Nicotiana glauca*) showed no symptoms of any kind except a general stunting of the plants, 1 (*N. glutinosa*) showed stunting and necrosis but no traces of mottling or chlorosis, and 1 (*S. laciniatum*) showed only a very faint mottling. Moreover, the inclusions, if present, were usually very abundant in the tissues and occurred in all parts of the leaf lamina except in the vascular bundles. In pepper (*Capsicum annuum*), however, where only a general chlorosis of the leaves was produced, without any distinct mottling, the striate material was abundant throughout the tissues, but the x-bodies were present only in the cells adjacent to the veins. In all hosts both types of inclusion were closely similar to those occurring in tobacco, resembling them in form, general appearance, staining properties and, except in pepper, in general distribution in the leaf. In some hosts, however, the x-bodies did not reach quite as large a size as in tobacco. It is interesting to note that *P. alkekengi*, once reported as a symptomless carrier (26), but which developed a definite mottling after inoculation with tobacco mosaic in the writer's trials, contained both x-bodies and striate material in the cells. Tobacco plants inoculated with a combination of tobacco and cucumber mosaic viruses, or with a combination of tobacco mosaic virus and six other viruses, also developed inclusions of these two types, as did tobacco plants inoculated with tobacco mosaic virus which had been attenuated by heat (12). Similar inclusions have been described by Goldstein (?) in *S. sculeatissimum* affected with tobacco mosaic. It is, therefore, evident that these two types of cell inclusion, namely, x-bodies and striate material, already known to be associated with tobacco mosaic on tobacco, must be considered as a constant feature of this disease irrespective of host plant, occurring wherever a definite mottling or chlorosis is produced.

On the other hand, no cell inclusions were observed in any healthy plant, although material from mosaic-free individuals of each host species under consideration was always examined at the same time as material from mosaic plants.



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## FORM-CLASS TAPER CURVES AND VOLUME TABLES AND THEIR APPLICATION<sup>1</sup>

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### I. GENERALIZED TAPER CURVES AND VOLUME TABLES

#### NEED FOR IMPROVEMENT OF TREE VOLUME TABLES

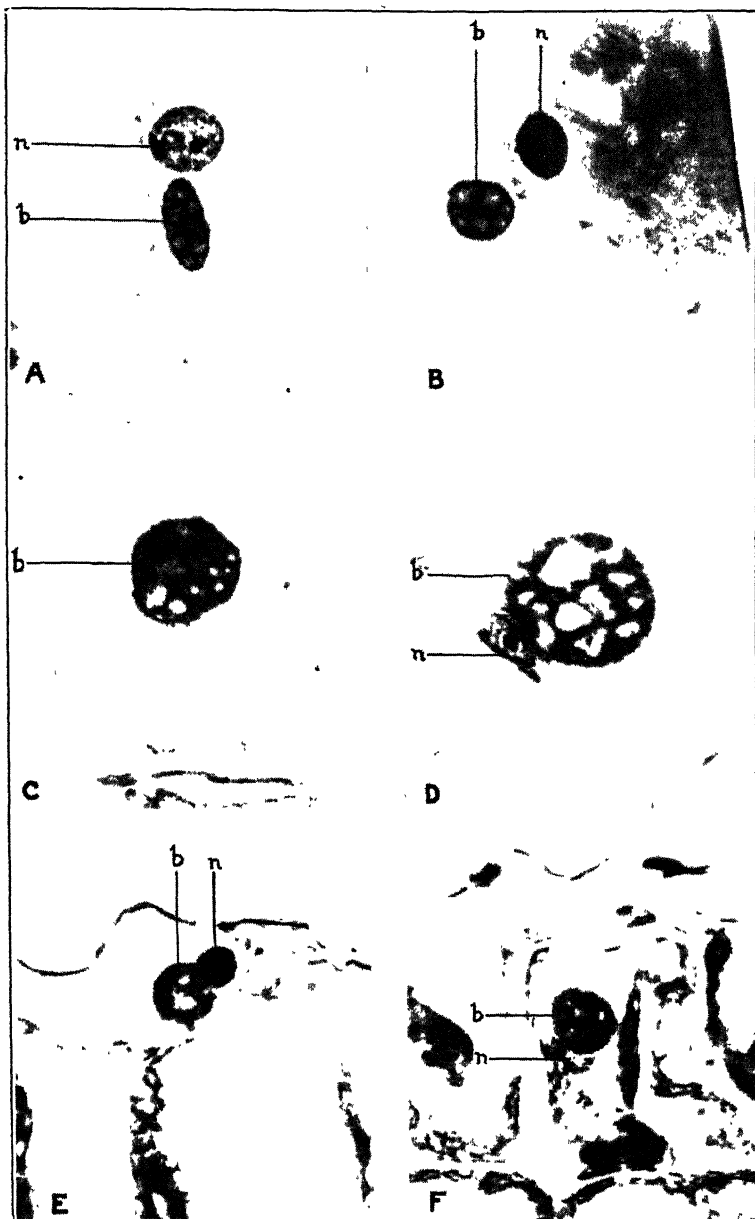
As an aid in determining the contents of stands of timber in timber cruising and growth studies, foresters are accustomed to use volume tables showing the average volume in a given unit of product of trees of different diameters and heights. These tables have generally been made up for each individual tree species, and for species occurring over a wide range of territory different sets of tables have often been constructed in different portions of their range. It is desirable to have tables which will be applicable over as wide a range as possible in order to eliminate the necessity of special studies for the construction of tables from local material for each individual forest or project. In the construction of tables for general application measurements have been made on large numbers of trees of a given species covering every possible condition in many localities and these have all been averaged together to give a single average volume for each diameter and height class. In spite of the enormous amount of work necessary for the construction of such tables, experience has shown that they are often not dependable for timber cruising nor for the more exacting demands of growth and yield studies. The work reported in this paper was commenced by the author at the School of Forestry, University of Idaho, in 1920, and the principal findings (3)<sup>3</sup> were reached at that institution before the author became connected with the United States Department of Agriculture, Forest Service, at the Northeastern Forest Experiment Station, in the fall of 1923. Under the auspices of the Forest Service the work was amplified and elaborated in preparation for its publication. The report is, therefore, a cooperative contribution from these two institutions.

The volume of a tree is dependent upon three factors—diameter, height, and form. Volume tables of the conventional type described above recognize only differences according to diameter and height and show volumes corresponding to the average form of the material

<sup>1</sup> Received for publication June 6, 1927; issued November, 1927.

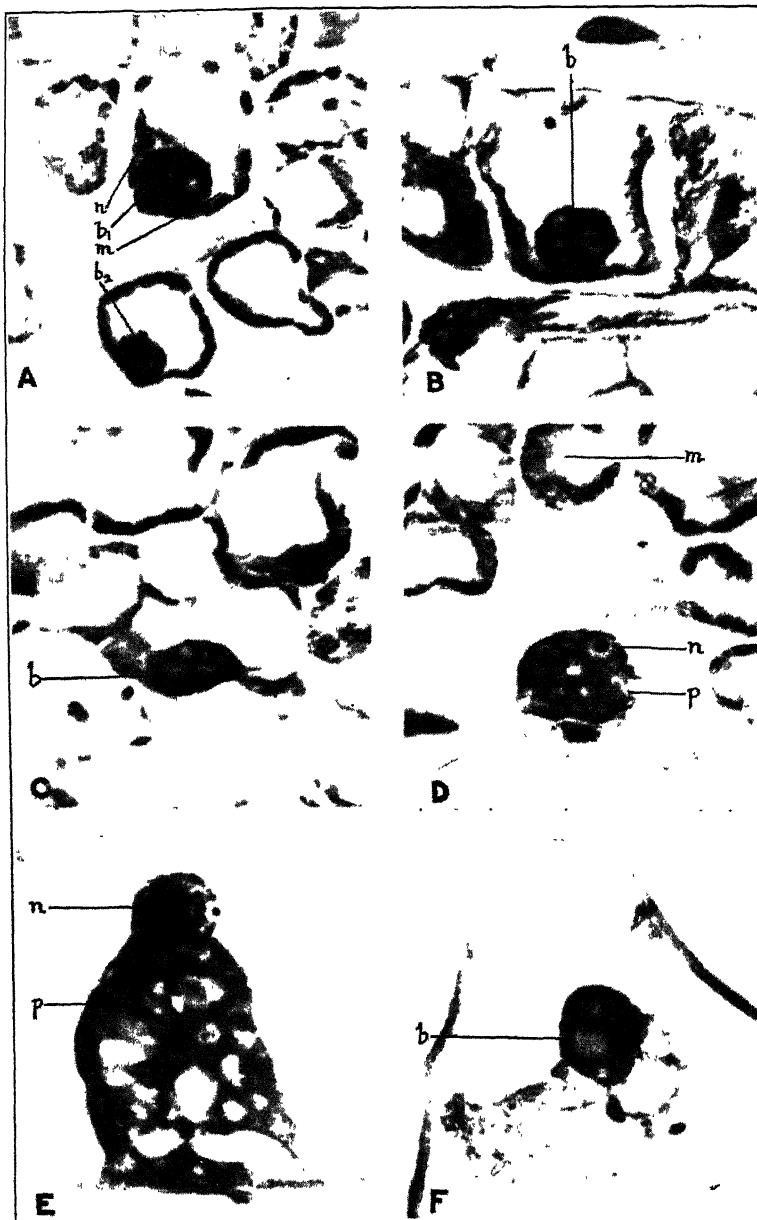
<sup>2</sup> The author wishes to acknowledge his indebtedness to J. Phillip Drissen, James Farrell, Harold Z. White, and Rodgers G. Wheaton, formerly students at the University of Idaho, for their share in the initial stages of the project, and to Donald Bruce, Joseph Kittredge, E. N. Munns, F. S. Baker, and Walter H. Meyer, of the U. S. Forest Service, for valuable suggestions in the preparation of the manuscript.

<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 743.



Photomicrographs of cell inclusions present in the leaves of certain mosaic-diseased plants.  
 X 785

- A.—Tobacco mosaic on tobacco. X-body (*b*) and nucleus (*n*) in a hair cell.
- B.—Tobacco mosaic on tobacco (nucleus slightly out of focus).
- C.—Tobacco mosaic on tobacco. Large x-body (*b*) in a hair cell. This was one of the largest observed in this type of material.
- D.—Yellow tobacco mosaic on petunia. Very large, coarsely granular body (*b*) in contact with slightly crushed nucleus (*n*).
- E.—Crinkle mosaic on Green Mountain potato. Body (*b*) and nucleus (*n*) in epidermal cell.
- F.—Crinkle mosaic on Green Mountain potato. Body (*b*) and part of nucleus (*n*) in palisade cell.



Photomicrographs of cell inclusions in the leaves of potato in association with certain virus diseases.  $\times 785$

A.—Crinkle mosaic on Green Mountain Potato. Bodies ( $b_1$  and  $b_2$ ), nucleus ( $n$ ) and brown material ( $m$ ) in mesophyll cell.

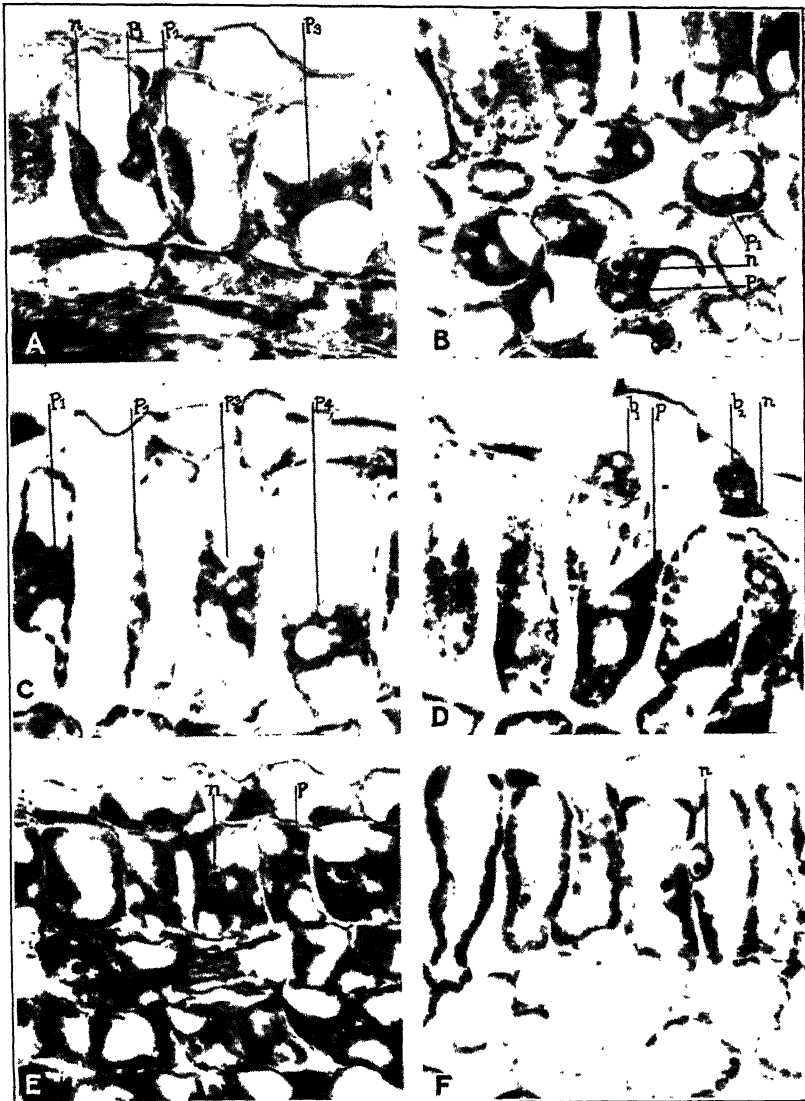
B.—Crinkle mosaic on Green Mountain potato. Body ( $b$ ) in palisade cell.

C.—Rugose mosaic on Green Mountain potato. Body ( $b$ ) in mesophyll cell.

D.—Rugose mosaic on Green Mountain potato. Mesophyll cell filled with vacuolate material ( $p$ );  $m$ , brown material.

E.—Supermild mosaic on American Wonder Potato. Large mass of vacuolate material ( $p$ ) and nucleus ( $n$ ) in hair cell.

F.—Tobacco mosaic on Rural New Yorker potato. X-body ( $b$ ) in hair cell.



Photomicrographs of vacuolate, protoplasmlike material in the leaves of potato in association with certain virus diseases.  $\times 590$

A.—Crinkle mosaic on Green Mountain potato. Vacuolate material in palisade cells as an irregular layer or mass adjacent to the wall ( $p_1$ ,  $p_2$ ), or as a bridge across the cell ( $p_3$ );  $n$  nucleus.

B.—Crinkle mosaic on Green Mountain potato. Vacuolate material in mesophyll cells, forming a layer adjacent to the wall ( $p_1$ ), or an irregular mass ( $p_2$ );  $n$  nucleus.

C.—Crinkle mosaic on Green Mountain potato. Vacuolate material in palisade cells as a bridge across the cell ( $p_1$ ,  $p_3$ ), a layer against the wall ( $p_2$ ), or a partially rounded mass ( $p_4$ ).

D.—Crinkle mosaic on Green Mountain potato. Vacuolate material as an indefinite mass ( $p$ ) or as definite bodies ( $b_1$ ,  $b_2$ );  $n$  nucleus.

E.—Rugose mosaic on Bliss Triumph potato. Abundant vacuolate material ( $p$ ) in the cells;  $n$  nucleus.

F.—Healthy Green Mountain potato. No vacuolate material present;  $n$  nucleus.



In contrast with tobacco mosaic, no inclusions of any type were found to occur in association with cucumber mosaic when inoculated to the same hosts as tobacco mosaic. Cucumber mosaic was studied altogether on 10 solanaceous host species (Table 1), in 7 of which x-bodies and striate material were produced in abundance by tobacco mosaic; in the case of cucumber mosaic, however, cell inclusions were entirely absent from the host tissues, although on many hosts quite as marked symptoms of mottling were developed as were produced by tobacco mosaic. For example, on young tobacco plants, cucumber mosaic caused a very striking chlorosis which was quite as intense as the chlorotic areas developed by tobacco mosaic on this host (fig. 1, B, C), yet although a considerable amount of material was examined, neither x-bodies nor striate material were ever observed in association with cucumber mosaic. Young cucumber plants affected with this mosaic were also examined, but again no inclusions were observed. In order to determine whether x-bodies or other cell inclusions were associated with this disease, but were very slow in developing, material was also examined from certain leaves of tobacco, pepper, and cucumber which had already shown marked mottling for several weeks. No inclusions, however, were observed. This point is of particular interest since it was formerly believed by some investigators that cucumber and tobacco mosaic were identical, owing to the fact that they frequently produced very similar symptoms on tobacco. Cytological considerations, however, support the evidence derived from other studies that these two diseases are due to two different viruses.

As with cucumber mosaic, no inclusions were found to occur in association with seven other virus diseases of solanaceous plants, namely, speckled tobacco mosaic, mild tobacco mosaic, spot necrosis, ring spot, bleaching mosaic, tomato stem necrosis, and petunia mosaic, although, with the exception of the last named, these diseases were studied on a variety of host species (Tables 1 and 2), and the symptoms of mottling produced were always quite definite and frequently very striking<sup>6</sup> (fig. 1, F, G, H). Petunia mosaic was studied only on tobacco; considerable material was, however, examined, and the evidence is therefore considered as conclusive in this case also.

In connection with these seven virus diseases, it may perhaps be maintained that negative evidence is never wholly convincing, and that inclusions may have been present in some tissues although not detected. It is believed, however, that by the methods employed this possibility has been eliminated. By the method of staining used, the x-bodies showed up conspicuously in the finished slides as blue-gray to deep blue-black objects. They presented a very different appearance from the host nucleus and could be readily distinguished from this, as is apparent from Plate 1, A, the bodies appearing of a homogeneous and uniformly stained material with vacuoles, and the nuclei as very granular, almost colorless bodies containing numerous dark chromatin granules, and immediately recognizable by the conspicuous, deep black nucleoli. The x-bodies were also readily distinguishable from the chloroplasts, which stained more lightly than

<sup>6</sup> In addition to the host plants shown in Table 1, spot necrosis was examined on *Solanum robustum* and no inclusions were found.

the former, besides differing in shape, size, and structure; and with a little practice it became possible to detect the x-bodies in the tissues, if present, with ease and rapidity. Similarly, the striate material was very conspicuous and could not be confused with any other constituent of the cell. Moreover, when inclusions were present at all in any material, even if occurring but rarely, several at least could be detected in almost any section examined, and with certainty in every slide. Hence, as it was customary to mount, on an average, 50 or more sections per slide and to prepare three or more slides from each portion of leaf material examined, the possibility of inclusions if present completely escaping detection becomes remote. It is admitted that in some cases it was not possible to secure more than one batch of material of a given virus on a given host, but since this included four or five leaf portions taken from different leaves and often from different plants, it is maintained that in nearly all cases the examination of material was adequate for a diagnosis. Usually, however, two or more batches of material were secured at different times from different plants, and in some cases a considerable amount of material was worked through. For example, in the case of cucumber mosaic on tobacco, 16 leaf portions were examined from 10 different plants, and in the case of spot necrosis on the same host, 10 leaf portions from 8 plants. Comparing this with material of tobacco mosaic on tobacco, 41 leaf portions of the latter were examined from 34 different plants and in every case both striate material and x-bodies were found present. It is, therefore, believed that, where material was examined and no inclusions were observed, such inclusions were definitely not present and were not merely overlooked.

X-bodies and striate material were found to be associated with two other virus diseases in addition to ordinary tobacco mosaic, namely, yellow tobacco mosaic (fig. 1, E) and medium tobacco mosaic. In the former case, out of 9 hosts examined,<sup>7</sup> 4 contained inclusions of both types, 4 contained striate material only, and 1 showed no inclusions, while in the latter, out of 5 hosts, 1 showed both types of inclusion, 3 striate material only, and 1 no inclusions. (Tables 1, 2.) The inclusions were closely similar to those associated with ordinary tobacco mosaic in all cases except one. On petunia, yellow tobacco mosaic sometimes produced vacuolate bodies of a relatively enormous size, appearing more coarsely granular than the regular tobacco mosaic bodies, and considerably larger than the largest of these, though other smaller bodies were also observed in other cells of the same material. One of these large bodies is shown in Plate 1, D, *b*, closely associated with the host nucleus *n*, which appears slightly crushed. The presence of inclusions associated with these two diseases could be correlated to a certain extent with the intensity of symptoms produced. On the other hand, yellow tobacco mosaic, for example, produced a very striking mosaic pattern on tobacco leaves (fig. 1, E), although no x-bodies could be detected in the tissues.

It would, therefore, appear from a cytological standpoint that these two diseases were closely related to, though not identical with, ordinary tobacco mosaic. Although a study of differential symptoms indicates that they are not identical, the property studies conducted

<sup>7</sup> In addition to the host plants shown in Table 1, yellow tobacco mosaic was examined on *Solanum gisymbifolium*, and striate material but no x-bodies were found.

by Johnson (13) also suggest a close relationship, since trials of the thermal death point, aging in vitro, and resistance to certain chemicals gave similar results for each virus. Since it has been shown that some viruses may be attenuated or modified under certain conditions (12, 1), and since yellow and medium tobacco mosaic were both originally obtained from tobacco plants growing in the field, it is conceivable that they may be naturally modified forms of the ordinary tobacco mosaic.

Generally speaking, cytological considerations are in favor of the view advanced by Johnson (11) that tobacco and other solanaceous plants may be affected by several different and distinct viruses.

A detailed investigation of the origin or development of the x-bodies in this group of plants was not attempted. However, by studying very young leaves, a few millimeters in length, taken from the tips of young tobacco plants affected with ordinary tobacco mosaic, it was possible to trace back the x-bodies to very small forms which appeared always in close contact with the host nucleus, irregular in shape, frequently crescent-shaped or somewhat triangular, staining similarly to the larger bodies, and appearing to consist of the same material, but without any vacuoles. Slightly larger bodies were also observed in which vacuoles were beginning to develop. These small bodies are evidently identical with certain forms described by Rawlins and Johnson (29) as appearing in the early stages of the disease and believed by these authors to be a possible young stage of the larger vacuolate forms. They also resemble early stages of the intracellular bodies present in mosaic corn plants as described by Kunkel (15).

## VIRUS DISEASES OF THE POTATO

### MATERIAL

The material for investigation of the potato virus diseases consisted of a number of different varieties of potato affected with one or more of the following diseases: Rugose mosaic, mild mosaic, supermild<sup>8</sup> mosaic, Montana crinkle, leaf-rolling mosaic, calico,<sup>9</sup> streak, leaf roll, crinkle mosaic, and tobacco mosaic. Tubers affected with each of the first eight diseases were obtained under these names from the department of botany and plant pathology, Oregon Agricultural Experiment Station; crinkle mosaic on Green Mountain potatoes was obtained from E. S. Schultz, United States Department of Agriculture; and rugose mosaic, on Bliss Triumph potatoes, was of Wisconsin origin. The tobacco mosaic virus was identical with that discussed in the previous section in connection with other solanaceous hosts and was secured on different varieties of potato by juice inoculation. These potato viruses were also transmitted to a limited extent to other varieties of potato by artificial inoculation. All plants except those inoculated with tobacco mosaic were grown in a cool greenhouse where the symptoms were usually distinct. Typical symptoms produced by three of the viruses studied are shown in Figure 3.

<sup>8</sup> The term "supermild" mosaic is used only tentatively in this paper.

<sup>9</sup> Possibly a virus disease.

## METHODS

The material was examined by methods similar to those described in the previous section, both in the living and in the fixed condition. Formol acetic alcohol was employed throughout as fixing fluid, the fixed sections being stained with iron-alum haematoxylin as before.

## RESULTS

The study of the potato virus diseases was complicated both by the inconsistencies in nomenclature of the different diseases, and by the difficulty in recognizing some of them on the basis of described symptoms, which constitute at present almost the only criterion. For example, "Montana crinkle," originally obtained on Rural New Yorker variety, when inoculated to Bliss Triumph potatoes, produced symptoms identical with those of rugose mosaic on this variety (fig. 3, D), and, according to Folsom (6), the name "crinkle," as sometimes used, is synonymous with rugose mosaic. On the other hand, the "crinkle mosaic" of Schultz and Folsom (fig. 3, C) is considered by these authors (30) as distinct from the "crinkle" of other systems of nomenclature. It was, therefore, considered impracticable to attempt a detailed study of the different virus diseases of this group upon different varieties of potato along the lines described in the previous section of this paper until some other more definite means of identification and classification of the viruses concerned has been established. However, since the occurrence of amoebalike, intracellular bodies has been reported by Smith (32) in association with mild mosaic<sup>10</sup> on Golden Wonder and President varieties of potato, it was believed that a study of such material as was already at hand would be of value; and, from cytological considerations, this group of diseases proved to be singularly interesting on account of their showing what are believed to be different stages in the formation of definite, vacuolate, protoplasmlike inclusions in the cells of the diseased tissues. These inclusions in their final stages appeared to be very similar to the bodies described by Smith. Smith, however, does not mention any other stages, which are, from the present standpoint, one of the most interesting features. Such bodies have been observed in association with so-called crinkle mosaic, rugose mosaic, Montana crinkle, leaf-rolling mosaic (fig. 3, B), mild mosaic, and supermild mosaic, and have so far been found in the chlorotic areas only of the leaves. They have been studied most fully in the case of crinkle mosaic on Green Mountain variety (fig. 3, C), and will be considered first in this connection.

In healthy Green Mountain potatoes, in all except the very young leaves, the cells of the palisade and mesophyll tissues were found to contain only a narrow layer of cytoplasm which lined the inner surface of the cell walls and in which were embedded the chloroplasts and usually the nucleus. (Pl. 3, F, and fig. 2, A.) In the palisade cells, the nucleus was occasionally suspended in a narrow bridge of cytoplasm extending across the cell near the center. The cells of the epidermis and vein parenchyma contained a similar cytoplasmic layer and nucleus, but very few or no chloroplasts. In the fixed material, the cytoplasm appeared frequently somewhat shrunken or perhaps

<sup>10</sup> The type of mosaic is not mentioned in his publication, but in correspondence Smith states that it was "what might be called mild mosaic."

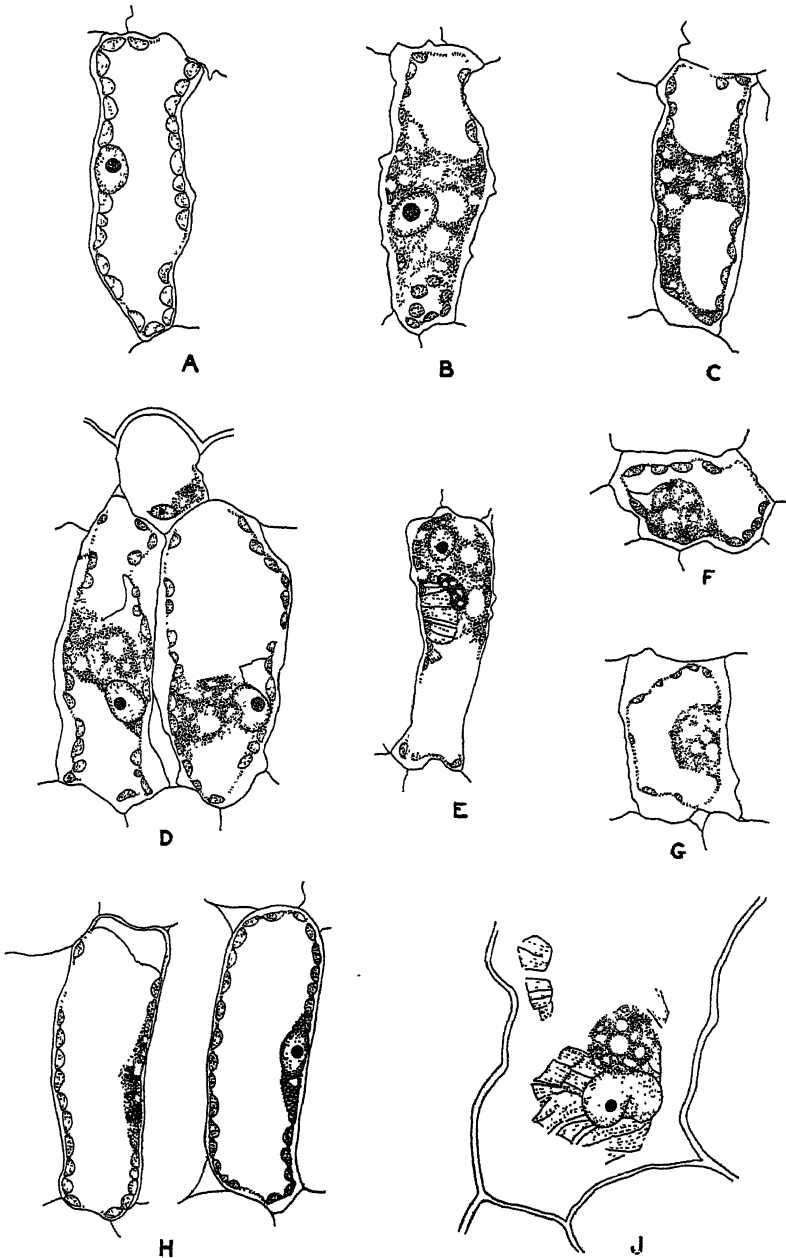


FIG. 2.—Cell inclusions in the leaves of potato in association with certain virus diseases.  $\times 700$ . A, Healthy Green Mountain potato. Palisade cell. B, Crinkle mosaic on Green Mountain potato. Indefinite mass of vacuolate material in palisade cell. C, Crinkle mosaic on Green Mountain potato. Vacuolate material as a bridge across palisade cell. D, Crinkle mosaic on Green Mountain potato. Vacuolate material in palisade cells and epidermal cell. Brown material in palisade cells shown in outline. E, Tobacco mosaic on Rural New Yorker potato. X-body, striate material, and vacuolate material in palisade cell. F, Crinkle mosaic on Green Mountain potato. Partially rounded mass of vacuolate material in mesophyll cell. G, Crinkle mosaic on Green Mountain potato. Body attached to host cytoplasm in parenchymatous cell adjacent to large vein. H, Rugose mosaic on Bliss Triumph potato. Small amount of vacuolate material adjacent to wall of palisade cell. Brown material shown in outline at top of left-hand cell. J, Tobacco mosaic on Rural New Yorker potato. X-body, striate material, and host nucleus in hair cell.

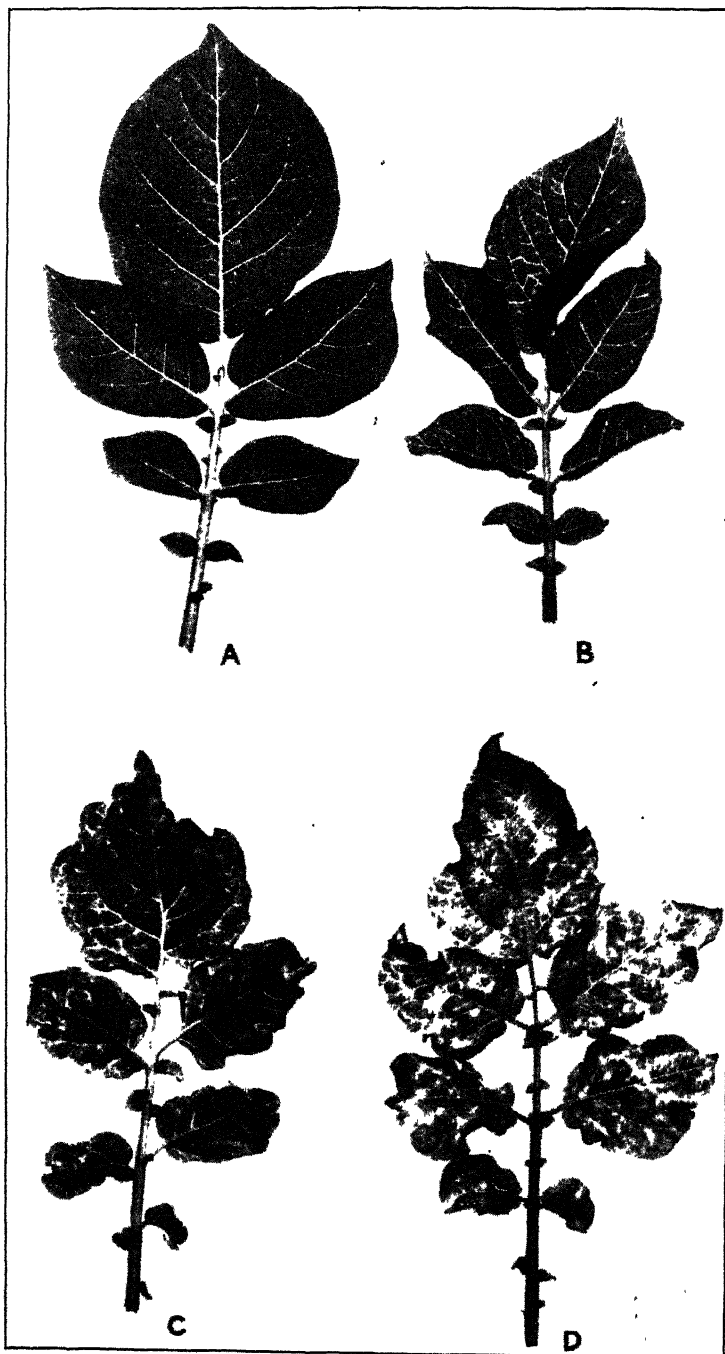


FIG. 3.—Types of leaf symptoms produced on potato by different virus diseases: A, Healthy Green Mountain potato; B, leaf-rolling mosaic on Green Mountain potato; C, crinkle mosaic on Green Mountain potato; D, rugose mosaic on Bliss Triumph potato

partially dissolved, and showed only as a very narrow layer. In plants affected with crinkle mosaic, on the other hand, in leaves on which the symptoms of mottling had become pronounced, many of the cells of the chlorotic areas were found to contain an excessive amount of fairly dense, vacuolate, protoplasmlike material, which was especially noticeable in the palisade and mesophyll cells. This material stained a blue-gray to deep blue-black shade with the iron-alum haematoxylin, and was present in varying amounts in different cells of the same tissue. In the palisade cells it was frequently seen as a bridge extending across the cell (pl. 3, A,  $p_3$ , C,  $p_1$ , and fig. 2, C, D), as a dark band or mass lying against one or more of the walls (pl. 3, A,  $p_1$ ,  $p_2$ , C,  $p_2$ ), or as an irregular, more diffuse mass, occupying a large portion of the cell (pl. 3, D,  $p$ , and fig. 2, B). In the mesophyll tissue it took the form of an irregular mass (pl. 3, B,  $p_2$ ) or a narrower band lying against the cell wall (pl. 3, B,  $p_1$ ). Similar material occurred in the epidermal cells and in the parenchymatous tissue surrounding the larger veins. In other cells of the same tissues the material appeared as if partially rounded up and approaching in form that of a definite "body." (Pl. 3, C,  $p_4$  and fig. 2, F.) In still other cells the same material was present but in the form of a quite definite, rounded mass or "body" (pl. 3, D,  $b_1$ ,  $b_2$ ) evidently similar to the "amoeba-like bodies" described by Smith. The dark green areas of the leaf, on the other hand, contained little or none of this vacuolate material. Portions of leaf material taken a week or more later from the same plant and from leaflets of about the same size were found to contain definite bodies of this type in the chlorotic areas in much greater abundance, while the irregular or indefinite forms of the same vacuolate substance were more rare. Such bodies are shown in Plate 1, E and F, and in Plate 2, A and B. The chloroplasts in the chlorotic areas were now found to be gorged with starch in both palisade and mesophyll tissues. The bodies were usually round or oval in shape, sometimes rather irregular in outline, but very definite. They were about twice as large in diameter as the host nucleus, and appeared to consist of a coarsely granular material containing one or more vacuoles. They were often situated in close contact with the nucleus, sometimes appearing attached to it, but at other times lying free. They seldom showed any clear indication of a limiting membrane, nor was any structure observed which could be interpreted as a nucleus, either in them or in the irregular vacuolate masses previously described. The bodies were somewhat similar to those of tobacco mosaic, but were on an average considerably larger; they usually contained fewer and larger vacuoles and were of a more coarsely granular nature. Occasionally, forms were observed which appeared to be drawn out at one point into a narrow projection resembling an appendage. The bodies often appeared to be attached to, or in close union with, the cytoplasmic layer of the host cell. (Fig. 2, G.) Material taken from leaves very soon after these had developed symptoms contained no definite bodies, though a certain amount of vacuolate material was present in irregular or indefinite forms. Since the definite bodies apparently do not occur in the tissues until somewhat later in the development of the disease, and since a series of intermediate forms can be found between the irregular masses of vacuolate material and the bodies, it is suggested that the

latter are formed by a process of rounding up of the vacuolate material in the host cells.

The bodies and other forms of vacuolate material were studied also in fresh, unstained tissues by means of free-hand razor sections mounted in water or in physiological salt solution. In this condition, the vacuolate material appeared similar in form and structure to that observed in the stained preparations. It was seen to consist of a colorless, hyaline substance, protoplasmic in appearance and closely resembling the cytoplasm of the cell, with which it was in apparent continuity. In the stained slides it was observed that, wherever plasmolysis had occurred in the process of fixation, the vacuolate material had drawn away from the cell wall together with the cytoplasmic layer (pl. 3, C,  $p_4$ ), indicating some kind of union with, or attachment to, the cytoplasm, and suggesting a common origin.

As to the nature of this material, it is not believed to be a degeneration product of the nucleus, as Smith suggested, since many of the cells which contain the substance, either as a definite body or as an indefinite mass, contain also an apparently normal nucleus. (Pl. 2, A, pl. 3, A, B, and fig. 2, B, D.) It is suggested, however, that it may be cytoplasmic in nature, representing some modification or abnormal form of the host cytoplasm.

Bodies and vacuolate material similar to those of crinkle mosaic have been observed also in association with rugose mosaic on Bliss Triumph, Green Mountain, and Early Ohio potatoes, with "Montana crinkle" on Rural New Yorker potatoes, with leaf-rolling mosaic on Green Mountain potatoes and with mild and supermild mosaics on American Wonder potatoes. Plate 2, C, shows a body in the leaf of Green Mountain potato affected with rugose mosaic, and Plate 2, D, a mesophyll cell of the same tissue completely filled with the vacuolate material. Plate 2, E, shows a large mass of vacuolate material in a leaf-hair of American Wonder potato affected with supermild mosaic. With the exception of "Montana crinkle" on Rural New Yorker variety, however, the bodies were of less frequent occurrence in all these cases, though in Bliss Triumph potatoes affected with rugose mosaic the vacuolate material was extremely abundant, occurring in almost every cell. (Pl. 3, E.) Here the nuclei frequently appeared at first sight as if partially dissolved or degenerating (pl. 3, E,  $n$ ), but a more careful examination showed that this was due to the partial concealment of the nuclei by the vacuolate material, and in cells containing definite bodies the nuclei were normal in appearance. In earlier stages of the development of this disease on Bliss Triumph variety, the vacuolate material was much less abundant and was apparently limited to narrow bands or masses lying against the wall of the cell, and containing few or no vacuoles. (Fig. 2, H.) In American Wonder variety affected with mild and supermild mosaics, the vacuolate material was not very abundant in the cells and definite bodies were of relatively rare occurrence. This may, perhaps, be correlated with the comparatively mild symptoms produced by these two mosaics. Similar vacuolate material, but no bodies, were observed also in association with supermild mosaic on Burbank, and with rugose mosaic on Cobbler, King, and Burbank varieties, and it is possible that, in some cases at least, definite bodies would have formed had the plants been kept for a sufficient length of time. On the other hand, no such vacuolate material was observed in association with

calico and leaf roll on Burbank or with streak on Bliss Triumph variety.

Further investigations of the origin and development of this vacuolate material are in progress.

A second type of inclusion was present in the stained material in association with all the virus diseases studied with the exception of calico, leaf roll, and streak. This consisted of a brownish substance occurring in irregular masses in the different tissues of the leaf, sometimes in cells which contained the vacuolate material, and sometimes in cells where this was not present. (Pl. 2, A, D, *m*, and fig. 2, D, H.) This substance was not observed in this form, however, in fresh, unstained tissues and its origin has not yet been determined. No striate material was found associated with any of these diseases of the potato.

On inoculation of the ordinary tobacco mosaic virus to potato (Rural New Yorker and Green Mountain varieties) both x-bodies and striate material appeared in abundance in the cells and were similar to those occurring in other host plants affected with this virus. (Pl. 2, F, and fig. 2, J.) In addition to these inclusions, many cells were found to contain irregular masses of vacuolate material similar to that associated with the potato mosaic diseases already described. This material stained somewhat more lightly than the tobacco bodies and was readily distinguishable from the latter on this account as well as by its irregular form and larger vacuoles. Frequently a single cell was found to contain a tobacco x-body and striate material as well as the vacuolate material. (Fig. 2, E.)

In the potato virus group, cytological evidence at present suggests that some, at least, of the viruses may be closely related, since vacuolate, protoplasmlike bodies of a similar nature are associated with several of these diseases. On the other hand, since no inclusions were found present in the case of leaf roll, calico, and streak, their production is evidently characteristic only of certain virus diseases of potato.

#### DISCUSSION

The results described in the preceding pages have a bearing both on the proposed classification of plant viruses (13) and on the nature of the x-bodies, or protoplasmic inclusions, present in certain mosaic-diseased tissues.

In the first place, it has not been found possible, as was originally hoped, to establish, to any great extent, any means of classification of viruses on the basis of microscopical features of the diseased tissues. It has, however, been shown that the production of cell inclusions in the tissues of various host plants is characteristic of certain viruses only, and must, therefore, be ascribed to some specific character or property of these viruses and not of the host plants. It follows that the presence of such inclusions may in these cases be employed as an additional means of identification of the viruses in question, and it is believed that in the future such features will become of more general application in the recognition of certain virus diseases in cases where a single host plant is susceptible to more than one virus. The need of some system of classification of plant viruses is already apparent and has been discussed in detail elsewhere (13), and it is believed that cytological features such as those now under consideration will prove

of value in supplementing other known diagnostic characters in any established system of classification.

The more fundamental considerations, however, are those which relate to the nature of the x-bodies or protoplasmic inclusions, which have been variously interpreted as protozoa or other living organisms and the causal agent of the disease in question, and as certain degeneration or reaction products of the host cells.

The constant association of these bodies with tobacco mosaic on various host plants is in harmony with the causal organism theory. It must, however, be remembered that the striate material is also a constant feature of the disease, yet this substance is crystalline in nature and can hardly be a form of living organism. On the other hand, if the x-bodies are of a causal nature, it is to be expected that they would be constantly associated with a large number of, if not all, mosaic diseases. Up to the present, they have been reported in connection with 10 different plant diseases, namely, mosaic of tobacco, corn, sugar cane, Fiji disease of sugar cane, mosaic of wheat, *Hippastrum equestre*, *H. johnsonii*, Chinese cabbage, potato, and dahlia. Few reports have yet been made of plant virus diseases with which such bodies are definitely not associated. McKinney (21) was unable to find definite inclusion bodies in the case of mosaic of cucumber, raspberry, or sweet clover, though he does not consider these negative results as final. Smith (31) recently states that intracellular bodies were not observed in mosaic Aquilegia and only rarely in pokeweed, though she gives no indication of the type of mosaic with which she was working. Evidence is presented in this paper of eight virus diseases of solanaceous plants with which such inclusions definitely do not occur, at least under greenhouse conditions. These are: Cucumber mosaic, speckled tobacco mosaic, mild tobacco mosaic, spot necrosis, ring spot, bleaching mosaic, tomato stem necrosis, and petunia mosaic. Neither have inclusions been observed so far in association with leaf roll, streak, or calico of potato. The mosaic of petunia described by Smith (31) which produced inclusion bodies and striate material similar to those of tobacco mosaic on tobacco is believed to be ordinary tobacco mosaic on petunia, and not the petunia mosaic as described by Johnson (11) and now under consideration. It is believed furthermore, that a number of other mosaic diseases will be found to show no inclusion bodies in the host tissues, and it is hoped that, in the future, investigators who look for such bodies will make a definite report even in the case of negative results. It is, of course, possible that all virus diseases are not due to a similar cause, and this appears probable when one considers diseases such as the "yellows" group on the one hand, and the "mosaic" group on the other. The diseases under discussion, however, for the most part come within the "mosaic" group, and it is more difficult to conceive of such diseases as tobacco and cucumber mosaic, for instance, as falling into different categories since these show so many striking resemblances.

While it is too early yet to draw final conclusions concerning the vacuolate bodies associated with the potato virus diseases, the evidence bearing on their origin is very suggestive. If these bodies actually represent some modification or abnormal form of the host cytoplasm, then it is likely that the x-bodies of tobacco and other mosaics are also products of diseased cells and not the causal agents of the diseases with which they are associated.

## SUMMARY

Two types of cell inclusion, namely, x-bodies and striate material, previously described in association with tobacco mosaic on tobacco, are found to be a constant feature of this disease on solanaceous host plants which develop definite symptoms of mottling or chlorosis.

Similar inclusions are associated with two other virus diseases of solanaceous plants, namely, yellow tobacco mosaic and medium tobacco mosaic, which appear from various considerations to be closely related to ordinary tobacco mosaic.

Eight other virus diseases are found to produce no inclusions of either type in any of the solanaceous hosts examined.

It is believed that the presence or absence of such inclusions will prove of more general application in the identification of certain different virus diseases in cases where a single host species is susceptible to more than one virus.

An abnormal, vacuolate, protoplasmlike material occurs in the cells of potato in association with several different virus diseases. This material appears in various forms, either as definite bodies or as irregular masses whose appearance suggests intermediate stages between the bodies and the host cytoplasm.

It is believed that the evidence presented in this paper is on the whole in favor of the view that the x-bodies are not of the nature of a causal organism.

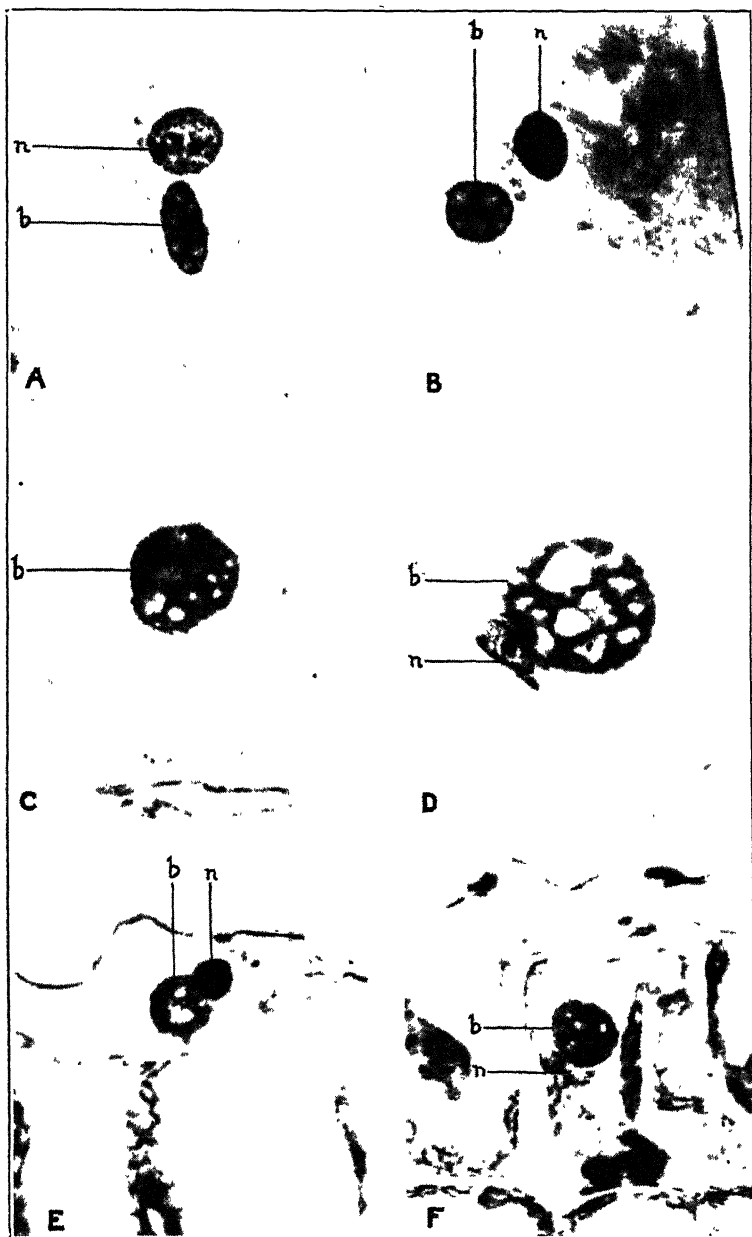
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Photomicrographs of cell inclusions present in the leaves of certain mosaic-diseased plants.  
 × 785

- A.—Tobacco mosaic on tobacco. X-body (*b*) and nucleus (*n*) in a hair cell.  
 B.—Tobacco mosaic on tobacco (nucleus slightly out of focus).  
 C.—Tobacco mosaic on tobacco. Large x-body (*b*) in a hair cell. This was one of the largest observed in this type of material.  
 D.—Yellow tobacco mosaic on petunia. Very large, coarsely granular body (*b*) in contact with slightly crushed nucleus (*n*).  
 E.—Crinkle mosaic on Green Mountain potato. Body (*b*) and nucleus (*n*) in epidermal cell.  
 F.—Crinkle mosaic on Green Mountain potato. Body (*b*) and part of nucleus (*n*) in palisade cell.

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## FORM-CLASS TAPER CURVES AND VOLUME TABLES AND THEIR APPLICATION<sup>1</sup>

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### I. GENERALIZED TAPER CURVES AND VOLUME TABLES

#### NEED FOR IMPROVEMENT OF TREE VOLUME TABLES

As an aid in determining the contents of stands of timber in timber cruising and growth studies, foresters are accustomed to use volume tables showing the average volume in a given unit of product of trees of different diameters and heights. These tables have generally been made up for each individual tree species, and for species occurring over a wide range of territory different sets of tables have often been constructed in different portions of their range. It is desirable to have tables which will be applicable over as wide a range as possible in order to eliminate the necessity of special studies for the construction of tables from local material for each individual forest or project. In the construction of tables for general application measurements have been made on large numbers of trees of a given species covering every possible condition in many localities and these have all been averaged together to give a single average volume for each diameter and height class. In spite of the enormous amount of work necessary for the construction of such tables, experience has shown that they are often not dependable for timber cruising nor for the more exacting demands of growth and yield studies. The work reported in this paper was commenced by the author at the School of Forestry, University of Idaho, in 1920, and the principal findings (3)<sup>3</sup> were reached at that institution before the author became connected with the United States Department of Agriculture, Forest Service, at the Northeastern Forest Experiment Station, in the fall of 1923. Under the auspices of the Forest Service the work was amplified and elaborated in preparation for its publication. The report is, therefore, a cooperative contribution from these two institutions.

The volume of a tree is dependent upon three factors—diameter, height, and form. Volume tables of the conventional type described above recognize only differences according to diameter and height and show volumes corresponding to the average form of the material

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<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 743.

from which they are constructed. Unless the form of the timber to be measured happens to average the same as the form of the trees from which the volume table was constructed it is evident that the table will not give a correct estimate of the volume of the timber. In attempting to include material from all possible conditions in the construction of volume tables the object is to get figures representing the true average form of the species in question, but in application, due to differences in density, age, site, or other factors, the timber over large areas may vary widely in form from the true average for the species and thus reduce the dependability of the tables for any particular stand. It has been recognized that differences in average form of the timber due to such local factors as those mentioned may cause more variation in volume for a given size class than a difference of a thousand miles in range.

In the construction of taper curves, which show the average diameter at any point on the stem for trees of different diameter and height classes, the same difficulty is encountered. Charts are constructed which attempt to show the average taper of the trees for any size class but no consideration is given to the fact that the actual tapers in a given stand may differ widely from this average.

In order to get dependable results in timber estimating, under all conditions, tables are needed which take into consideration the factor of form. In studying the growth of young timber it is found that volume tables made up from measurements of trees in virgin stands do not give reliable results. Tables differing from those for forest-grown material may also be necessary for timber grown in open old fields. In some species at least the growth of trees left after logging is known to be distributed over the stem in a manner differing from that in the original stands, and thus volume tables based on measurements of trees in the original stand may not be reliable in measuring the development of the residual crop. All these difficulties in the application of the conventional volume tables based on diameter and height alone are due primarily to differences in average form and may be more effectively met by a system which recognizes the factor of form in the basic tables.

The factor of form may be considered as the product of three components. In the first place, the shape of the main portion of the stem above the butt swell must be considered by itself. This is expressed by the type of form curve exhibited and is usually described under the categories of neiloidal, conical, or paraboloidal, although it will be shown that trees more generally approximate a hyperboloid in shape.

In the second place, trees vary in rate of taper independent of the type of form curve they exhibit. This is expressed by differences in form quotient or absolute form factors, and timber is usually described in respect to this component as full-boled or rapid in taper.

Finally, trees differ markedly in bark thickness and amount and upward extension of butt swell. This is important in timber estimating because the accepted standard for measuring standing timber is the diameter outside bark at breast height. For trees of the same shape and rate of taper in the main portion of the stem, the wood volume will vary for a given breast-high diameter, according to the thickness of bark and amount of butt swell at breast height.

## DEVELOPMENT OF THE FORM-CLASS SYSTEM IN SWEDEN

In Sweden, form-class volume and taper tables have for a number of years been used as the basis for timber estimating. In these tables the trees are classified not only by diameter and height but also by form quotient, which is defined as the ratio between the diameter at one-half the height above breast height and the diameter at breast height. The form quotient, originally proposed by Schiffel (25) and later modified by Jonson (13) is the best expression of tree form yet developed.

Previous to Jonson's work, Schiffel (25) and Maass (18, 19) had demonstrated that for trees of a given height and form class, taper was independent of diameter. In 1910 and 1911 Tor Jonson, working with Norway spruce (*Picea excelsa*) and Scotch pine (*Pinus sylvestris*), showed that taper for trees of a given form class was independent of height as well as diameter. His work also indicated that different species of conifers, and possibly of hardwoods as well, might be measured by the same set of generalized taper curves, since it is reasonable to assume that all species taper in accordance with the same fundamental growth principle and that many species might express this in similar manner.

The form-class taper and volume tables now in general use in Sweden were computed by Tor Jonson from a formula developed by A. G. Höjer in 1903 (10) from measurements of Norway spruce. This formula gives the diameter at any point on the stem in percentage of diameter at breast height. It has the form  $\frac{d}{D} = C \log \frac{c+l}{c}$  in which  $D$  is the diameter at breast height,  $d$  is the diameter at distance  $l$  from the tip, and  $C$  and  $c$  are constants varying with the form quotient of the timber. As  $l$  is expressed in percentage of the total height above breast height, the equation is general and applicable to trees of any size class.

Tor Jonson (13, 14) showed that Höjer's equation was in close conformity to the actual tapers of Norway spruce, but in comparing it with the actual tapers of Scotch pine he noted a considerable falling off in the tops, which he attempted to correct by the introduction of a so-called "biologic" constant in the original equation, making it read  $\frac{d}{D} = C \log \frac{c+l-2.5}{c}$  for Scotch pine. In spite of this failure of Scotch pine to conform exactly to the original equation, Jonson decided that the deviation was not so serious but that the volume tables would be sufficiently accurate for this species as well as Norway spruce.

In using the form-class tables no attempt is made to estimate the form of each individual tree, but an estimate is made of the average form of the stand and the table for the corresponding form class is used for all the trees in the stand. The use of a single average form quotient for the entire stand is based on the proposition that most of the trees in the stand will have very nearly this average form and that the trees having a better form will be offset by those having a poor form. The validity of this proposition is a subject for investigation.

It is difficult to determine the average form quotient of the standing timber by actual measurement of the middle diameters of the

trees, but by the use of the form-point idea Jonson (15) developed an indirect method of estimating the average form of the timber. As a basis for the form-point method, Jonson assumed that the wood is laid on according to the stress to which the stem is subjected in supporting the crown, and that the wind, exerting its pressure against the crown, is the chief agent in setting up stresses in the stem. The distribution of the stresses in the different portions of the stem, which results in differences in form of the trees, therefore, depends upon the size, shape, and position of the crown. The form point is defined as that point in the crown where the wind pressure may be considered as concentrated, which usually will correspond to the center of gravity of the area presented to the prevailing wind. The relative height of the form point in the tree is thus an index of the length, size, and shape of the crown, and therefore an index of the form of the stem. The form-point height is expressed as percentage of the total height of the tree and is measured very rapidly with the use of a Christen hypsometer divided into 10 equal divisions. Jonson worked out a table showing the form quotient corresponding to different form-point heights for Norway spruce of different total height classes. He also found that density is a good indicator of the average form of the stand, and suggests that with a little practice the average form of a stand can be judged very well on the basis of its density.

Although Jonson's form-point idea is apparently giving satisfactory results in practical use in Sweden, the wind-pressure theory upon which it is based is by no means universally accepted. Hohenadl (11) presents convincing argument for the theory that the tree stem is built up in such a manner as to best support its own weight rather than to resist lateral bending from wind pressure. According to Hohenadl the diameter at any point on the stem is a function of the weight of the tree above that point and the mechanical strength of the section at the point in question. The form-point idea might be adapted to this conception just as well as to the wind-pressure theory. For trees of average development there would be little difference in the relative height of the form point according to the two theories. The fact that little difficulty has been experienced in the use of the form point in Sweden does not, therefore, either prove or disprove the correctness of Jonson's conception.

Another factor which enters into the application of these tables is the effect of the bark thickness and butt swell extending above breast height upon the form quotient of the timber. The formula used in computing the tapers applies for diameters inside bark and takes no account of any inflection of the stem curve toward the base of the tree which results whenever butt swell extends above breast height. The basis for application of volume tables is commonly the diameter outside bark at breast height so that different form quotients as well as different volumes will be indicated for the same d. b. h.<sup>4</sup> class when there are differences in thickness of bark or amount of root swell at the breast height point.

For the thin-barked Norway spruce, Jonson (13) found the form outside bark to be essentially the same as the form inside bark; but for Scotch pine (14) he found great differences in bark thickness

<sup>4</sup> D. b. h. = diameter at breast height.

in the timber from different portions of the country, which made the form outside bark poorer in varying degrees than the form inside bark. He also noted that when the root swell extended above breast height the indicated form quotient was lower than the actual form of the tree, but apparently this distortion was not general and Jonson suggests no satisfactory method of dealing with it.

Mattsson (20) has made a detailed study of the form and form variations of European and Siberian larch. He found their tapers almost identical and very similar to the form of Scotch pine as worked out by Jonson, although he noted a falling off in the upper sections of the tall trees in the larger form classes. The bark thickness was found to vary greatly in different stands but these differences could not be correlated with any fixed character of the stand but rather seemed to be due to heredity or a specialization of the species. Investigations in growing trees from seed from different countries substantiated this theory.

Mattsson found that the butt swell in larch reaches breast height in trees 10 meters tall and mounts steadily with increasing height. He reached no definite conclusions as to the factors affecting butt swell. He found the total range of form class within a stand to be 25 to 27 form class units, but the trees distributed themselves about the average in accordance with the normal probability curve. The variation of the form class within a stand could not be connected with any special character of the stand nor with the diameters of the trees. It was found that the variation of the form quotient in trees of the average diameter class was about as great as the total variation in form quotient in the entire stand.

Mattsson found a close correlation between average height of the stand and average form class, and in the few cases where estimates of the average form according to average height of the stand were not satisfactory the deviations seemed to be correlated with the density. He concluded that the average form class of the stand could be used with plenty of accuracy for practical work if it is judged on the basis of density and height or on height alone. Estimation of the average form class in this way gives more certain results than by the measurement of a number of sample trees unless the number of sample trees is more than forty or fifty.

From studies of form in fully stocked pine stands (21) Mattsson also concluded that a single average form class could be used for the entire stand. In this case he stated that the average form could be based on direct measurement of a number of sample trees representing a range of sizes in the stand. The variation interval of the form class within these stands was found to be 24 form-class units or about the same as for larch. Since in the fully stocked stands of pine the form class was found to increase with age, a table giving the average form class at different ages for stands of different densities is suggested for practical use in approximate estimation of the stand. From a study of the use of the form point for determining the average form class of the stand Mattsson found no difficulty in determining the form point in pine forests. He found that the difference in the estimate of form point by different persons was not more than 1 or 2 per cent. He concluded that with Jonson's form-point table the form class is generally estimated too low by about two form-class units. Petrini (23) found that Jonson's form-point function gives

an estimate of form class of Norway spruce which is too low by exactly the same amount. Both Mattsson and Petrini emphasized the fact that the form point gives the stand's average form class and not the form class of the individual stem.

Petrini (24) investigated the factors affecting the accuracy of form-class methods in Lapland pine. He found the bark thickness increasing directly with the diameter of the trees, the ratio between bark thickness and diameter breast high being 11.4 per cent. This relation appeared to be constant in all stands covered by the investigation. The root swell extended above 10 per cent of the trees' height in some instances and seldom averaged more than 3 per cent of the diameter breast high in any stand. It begins to affect the breast-height diameter in trees about 7 inches in diameter and increases rapidly with increasing diameter of the trees. Jonson's form-point function gave form-class values for pine running as much as 6.5 form-class units below the true form of the material.

In the fall of 1920 the writer began to investigate the applicability of the Swedish form-class system to American species. In the present paper it will be shown that many American commercial conifers are essentially alike in their taper in the main portion of the stem, and that the generalized equation of the stem curve can be expressed more exactly by a much simpler formula than that used in Sweden. From this it follows that if suitable methods of allowing for bark thickness and butt swell and of estimating the average form of timber in the field are developed, a single basic set of form-class taper curves and volume tables can be used for all species known to conform to the formula.

#### DEVELOPMENT OF NEW FORMULA FOR THE STEM CURVE FROM WESTERN YELLOW PINE MATERIAL

Measurements of the taper of 201 western yellow pines (*Pinus ponderosa*) in north Idaho were collected as a basis for testing the conformity of this species to Höjer's (10) equation, upon which the Swedish volume tables are based. This material included trees ranging in age from 30 years to overmaturity, and in size from 4 inches to 44 inches d. b. h. Trees were selected from stands of various types and on various sites. Very open grown as well as normal and overcrowded trees were included. In taking these measurements the breast-height point was first carefully located. Then the height from this point to the tip was measured with a tape, great care being taken to locate and measure the true tip whenever this had been broken off by the fall of the tree. The average diameter outside bark at the stump, breast height, and each tenth of the stem above breast height was then determined by measuring in two directions with a caliper. At each point of measurement the average bark thickness was measured by chopping into the tree on opposite sides of the stem.

The taper of each tree was then plotted, height above ground on diameter inside bark, in order to study the root swell and eliminate any effect it might have on the breast-height diameter. Wherever the reverse curve of the root swell extended above breast height the normal convex curve of the upper portion of the stem was continued downward to breast height, and from this was read what is called the "normal" diameter of the tree. The accuracy with which this

prolongation of the stem curve is accomplished is subject to a graphic check in the averages as described on page 736. This check can be applied to the first few stems plotted to serve as a guide for handling this part of the procedure.

Each of the upper diameters was then divided by this normal diameter with the slide rule, thus expressing the tapers of the tree in general terms of percentage of normal breast-height diameter for different percentages of the height above breast height. In this form the tapers of trees of all diameters and heights can be averaged together on the basis of form and the results compared to the values given by a generalized equation of stem form. The trees were grouped into form classes on the basis of their "normal" form quotient, as contrasted to the actual form quotient which would be obtained without correction for root swell. These average percentile tapers are presented in Table 1.

TABLE 1.—*Percentile tapers of western yellow pine*

Form class	Percentage of length from tip to breast height									Basis num- ber of trees
	90	80	70	60	50	40	30	20	10	
	Percentage of "normal" d. i. b.* at breast height									
45-----	89.7	76.1	68.9	55.7	46.6	36.2	26.0	17.7	10.0	3
55-----	92.2	83.3	74.3	63.5	54.8	45.8	34.5	24.2	12.7	6
60-----	93.8	85.5	78.2	69.8	59.9	50.0	37.8	27.3	15.1	17
65-----	93.5	88.6	82.6	75.5	65.7	56.5	44.6	31.0	18.8	29
70-----	95.2	90.5	85.1	78.7	70.6	60.9	50.2	36.5	21.2	55
75-----	95.9	91.8	87.2	81.3	74.9	66.3	55.3	40.7	23.2	70
80-----	96.7	93.9	90.7	85.2	79.1	70.3	59.4	44.4	25.7	18
85-----	97.5	95.1	92.6	87.5	83.5	74.9	61.2	45.4	33.1	3

\* D. i. b. = diameter inside bark.

The figures in Table 1 are the raw averages of the material, and it will be noted that direct comparison with Jonson's table of values from Höjer's equation for the different form classes is impossible because in no case does the raw average fall exactly on the class average to which it belongs. By harmonizing the raw averages through a system of plotting and replotting, values for the even form classes could be interpolated; but this is not necessary, as a more satisfactory comparison can be made from the raw averages by plotting them along with the values from Jonson's table as in Figure 1, with form classes as abscissae, upper diameters expressed as percentage of normal diameter breast high as ordinates, and a curve for each tenth of the stem above breast height. Values derived from a new equation, to be discussed presently, are also shown in Figure 1. It is evident from Figure 1 that in the lower form classes western yellow pine is quite consistent with Höjer's equation, but that in the larger form classes the diameters are much below the values from the equation in the upper portions of the stem and somewhat above them in the lower portions.

Jonson's figures for Scotch pine fell below Höjer's equation in the upper sections of the larger form classes in the same manner. His modification of the equation by the use of the "biologic" constant 2.5 gave him better results. This constant lowers the curve for the

section  $x=10$  in Figure 1 about three units in all form classes without materially changing its shape, and this effect so rapidly decreases in the sections below this that it is negligible in the lower half of the stem. In this way the values in the upper form classes are not reduced sufficiently and the values for the upper sections of the lower form classes (appearing in the lower left portion of the chart) are thrown too low. Increasing the biologic constant gives no better result.

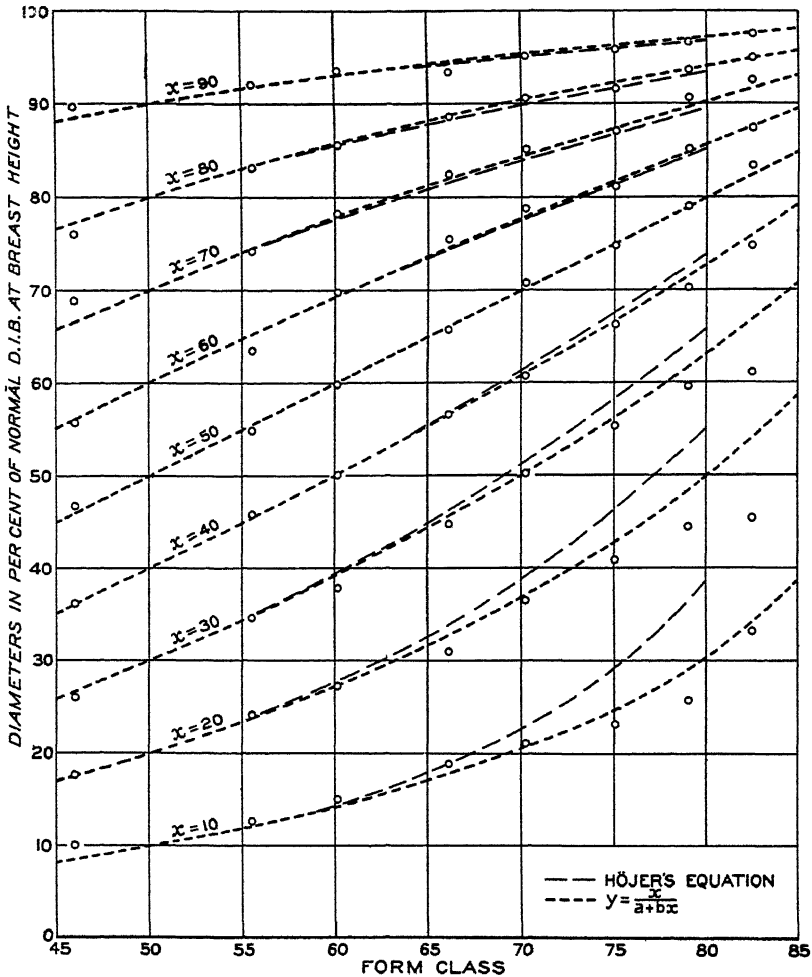


FIG. 1.—Percentile tapers of western yellow pine compared with formula values

When the biologic constant is 7.5 a good fit is obtained for form class 75 in all except the top section and form class 80 is somewhat improved, but the conformity of the lower form classes is entirely destroyed. The biologic constant makes a practically uniform reduction in the upper diameters of all form classes, whereas the variations noted in nature are confined to the higher form classes. Furthermore, increasing the value of the biologic constant exagger-

ates the inconsistency of the equation to a degree that can not be overlooked. This inconsistency lies in the fact that with the biologic constant the equation gives no diameter to that portion of the top of the tree represented by this constant. For small values like 2.5 per cent this is not serious, but the curve becomes entirely incompatible with nature if the value must be increased as high as 7.5 per cent.

Since the average values of the tapers of trees fall very nearly on a smooth curve, it seems probable that a relation does exist which may be represented mathematically by the equation of a curve, and if all trees lay on their growth in accordance with the same law such an equation ought to apply quite generally among different species. It seems evident that Höjer's equation fails to meet this requirement, as Norway spruce appears to be the only species approaching its value in the larger form classes. An attempt was therefore made to develop with the western yellow pine material an equation which would better express the normal taper of forest trees.

Graphic methods as presented by Lipka (17) were used in this analysis. The form of the curve of the experimental material gives some hint of the nature of the equation. It is desirable to choose an equation of the simplest possible form, the correctness of the form being tested by "rectifying" the curve, i. e., by rewriting the assumed equation in the form of a straight line in certain functions of the original variables. The data are then plotted with these functions of the original variables as coordinates; if the points of this plot appear to be on, or very near a straight line, then this line can be represented by the "rectified" equation, and hence the original curve by the assumed equation. When satisfied with the form of the equation chosen, it is necessary to determine values for the constants or coefficients appearing in the equation. The results should then be checked by new experimental material.

TABLE 2.—Ratio of percentage of length from tip to percentage of normal d. i. b.<sup>a</sup> at breast height= $x/y$ ; western yellow pine

Form class	Percentage of length from tip to breast height= $x$								
	90	80	70	60	50	40	30	20	10
	Values of $x/y$								
45.....	100.3	105.1	101.6	107.7	107.3	110.5	115.4	113.0	100.0
55.....	97.6	96.0	94.2	94.5	91.2	87.3	87.0	82.6	78.7
60.....	95.9	93.6	89.5	86.0	83.5	80.0	79.4	73.3	66.2
65.....	96.3	90.3	84.7	79.5	76.1	70.8	67.3	64.5	53.2
70.....	94.5	88.4	82.3	76.2	70.8	65.7	59.8	54.8	47.2
75.....	93.8	87.1	80.3	73.8	66.8	60.3	54.2	49.1	43.1
80.....	93.1	85.2	77.2	70.4	63.2	56.9	50.5	45.0	38.9
85.....	92.3	84.1	75.6	68.6	59.9	53.4	49.0	40.1	30.2

<sup>a</sup> D. i. b.=Diameter inside bark.

After a number of trials with equations of other types, the equation of the form  $y = \frac{x}{a + bx}$ , which represents an hyperbola, was found to correspond quite well with the western yellow pine material. This equation may be written  $x/y = a + bx$ , whereupon it becomes an equation of the first degree in  $x/y$  and  $x$ , and plotting with  $x/y$  and  $x$  as

coordinates should give a straight line. In this equation  $x$  corresponds to  $l$  of Höjer's equation and  $y$  to  $d/D$ ,  $a$  and  $b$  being constants varying with the form class. The variable  $y$  may, therefore, be defined as the ratio of the diameter at distance  $x$  from the tip to the

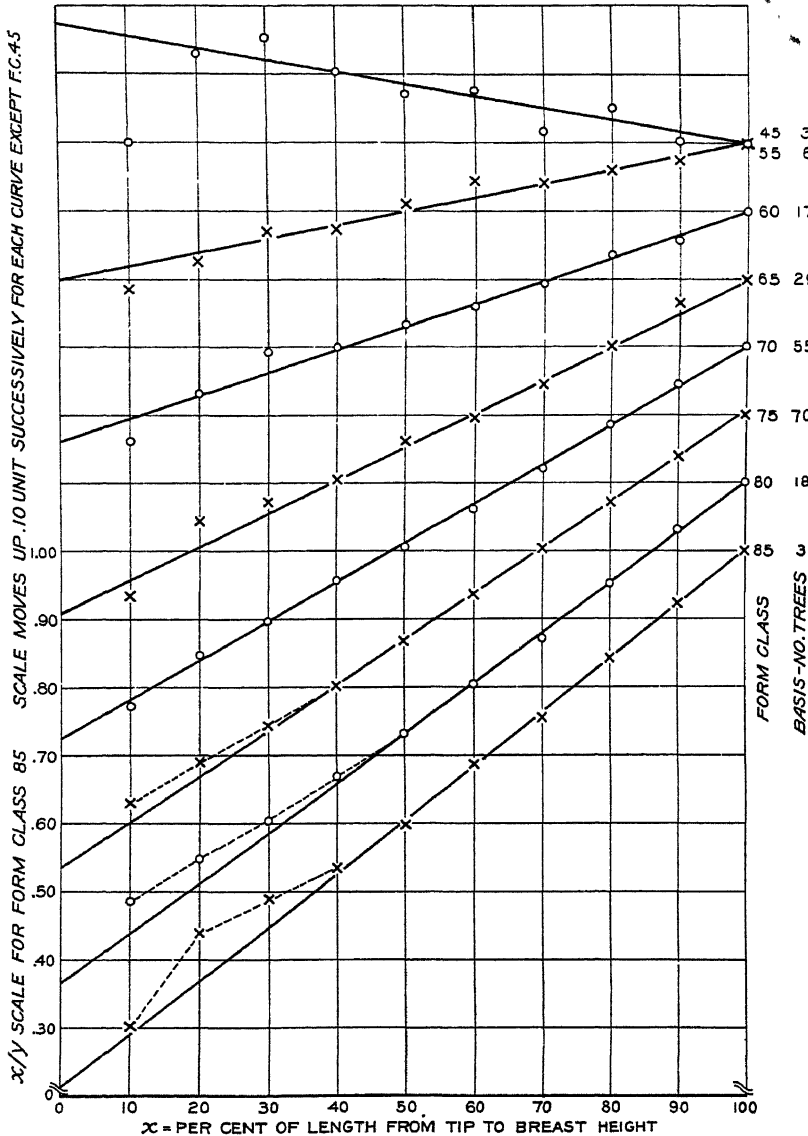


FIG. 2.—Plotting of  $x/y$  on  $x$  showing conformity to  $y = \frac{x}{a+bx}$  from data on western yellow pine, 201 trees, north Idaho

normal diameter at breast height, the distance  $x$  being expressed as percentage of the total height above breast height. Table 2 gives the corresponding values of  $x$  and  $x/y$  for the western yellow pine material as computed from the data in Table 1.

Figure 2 shows how nearly these values fall into straight lines when plotted. The scale of ordinates is moved downward 0.10 unit for each form class after 55 in order to keep the lines apart and present them more clearly. It will be noted that form classes 70 and 75, which have the best basis in number of trees, fit almost perfectly, and that the deviations in all the lower form classes are not consistent, but seem rather to indicate erratic averages, resulting from an insufficient number of trees. In form classes 80 and 85, however, there seems to be a definite tendency for the values of  $x/y$  for the upper sections to diverge upward from the straight line which fits the lower portion of the stem. This means that the actual diameters fall below the values given by the formula in these sections.

It should be emphasized that this equation, as well as that developed by Höjer, is entirely empirical, being an attempt to fit a curve to actual tapers as found in the field and is in no way dependent upon the fundamental theory of tree growth. No satisfactory formula has yet been proposed derived directly and deductively from the fundamental theories of tree growth.

#### TEST OF NEW EQUATION FOR OTHER SPECIES

With an equation satisfactorily representing the stem curve of western yellow pine, material for other species was examined to determine whether their taper could be measured by the same type equation. Table 3 summarizes data which were available for this test.

TABLE 3.—Material used in comparative tests

Species	Source and locality	Number of trees	Number of series
Norway spruce, <i>Picea excelsa</i> <sup>a</sup> .....	Jonson, 1910, Sweden.....	47	3
Scotch pine, <i>Pinus sylvestris</i> <sup>a</sup> .....	Jonson, 1911, Sweden.....	94	4
European larch, <i>Larix europea</i> <sup>b</sup> .....	Matsson, 1917, Sweden.....	503	12
Siberian larch, <i>Larix sibirica</i> <sup>b</sup> .....	do.....	229	3
Western white pine, <i>Pinus monticola</i> , <sup>b</sup> <sup>c</sup> .....	Behre, 1923; U. S. F. S., 1910-1913, north Idaho.....	139	6
Do. <sup>b</sup> .....	Cloughton-Wallin, and McVicker, 1920, British Columbia.(?).....	16	1
Douglas fir, <i>Pseudotsuga taxifolia</i> <sup>b</sup> .....	U. S. F. S., 1910-1913, North Idaho.....	72	5
Do. <sup>b</sup> .....	Cloughton-Wallin, and McVicker, 1920, British Columbia.....	11	1
Norway pine, <i>Pinus resinosa</i> <sup>c</sup> .....	Cloughton-Wallin, and McVicker, 1920, Ontario.....	81	3
Do. <sup>b</sup> .....	Reed, 1924, Massachusetts and New Hampshire.....	36	2
Northern white pine, <i>Pinus strobus</i> <sup>c</sup> .....	Cloughton-Wallin, and McVicker, 1920, Ontario (young stands).....	29	3
Do. <sup>a</sup> <sup>c</sup> <sup>d</sup> .....	Wright, 1923, Ontario (old stands).....	342	9
White, red, and black spruce <sup>a</sup> <sup>c</sup> <sup>d</sup> .....	Wright, 1923, Canada.....	865	8
White spruce, <i>Picea glauca</i> <sup>c</sup> .....	Cloughton-Wallin, and McVicker, 1920, Ontario.....	6	1
Red spruce, <i>Picea rubra</i> <sup>b</sup> .....	U. S. F. S., 1902, Piscataquis Co., Me. (virgin stands).....	132	4
Do. <sup>b</sup> .....	U. S. F. S., 1903, New Hampshire (second growth).....	20	1
Do. <sup>b</sup> .....	U. S. F. S., 1903, New Hampshire (culled forest).....	44	3
Do. <sup>b</sup> .....	U. S. F. S., 1903; Meyer, 1924, New Hampshire and Maine (old field).....	92	6
Balsam fir, <i>Abies balsamea</i> <sup>a</sup> <sup>c</sup> <sup>d</sup> .....	Wright, 1923, Canada.....	362	4
Do. <sup>b</sup> .....	Meyer, 1923, Maine (second growth).....	18	1
Do. <sup>c</sup> .....	Behre, 1924, Franklin Co., Me. (culled forest).....	20	1
Do. <sup>c</sup> .....	Behre, 1924, Franklin Co., Me. (old growth).....	73	5
Longleaf pine, <i>Pinus palustris</i> <sup>a</sup> <sup>b</sup> .....	Brown, 1924, southern United States.....	663	3
Shortleaf pine <i>Pinus echinata</i> <sup>a</sup> <sup>b</sup> .....	do.....	416	3
Loblolly pine, <i>Pinus taeda</i> <sup>a</sup> <sup>b</sup> .....	do.....	338	3
Gray birch, <i>Betula populifolia</i> <sup>b</sup> .....	Hawley, 1923, Pennsylvania.....	177	6
Eastern hemlock <i>Tsuga canadensis</i> <sup>c</sup> .....	Merrill and Hawley, 1923, Connecticut.....	55	3

<sup>a</sup> No attempt to eliminate root swell in the averages.

<sup>b</sup> Trees measured at arbitrary intervals in the field, diameters at one-tenth intervals interpolated graphically.

<sup>c</sup> Trees measured at one-tenth intervals in the field.

<sup>d</sup> Harmonized and interpolated to even form classes.

Figures 3 to 15 show the plotting of  $x/y$  on  $x$  for these data either taken directly from the various authors or interpolated from their figures.

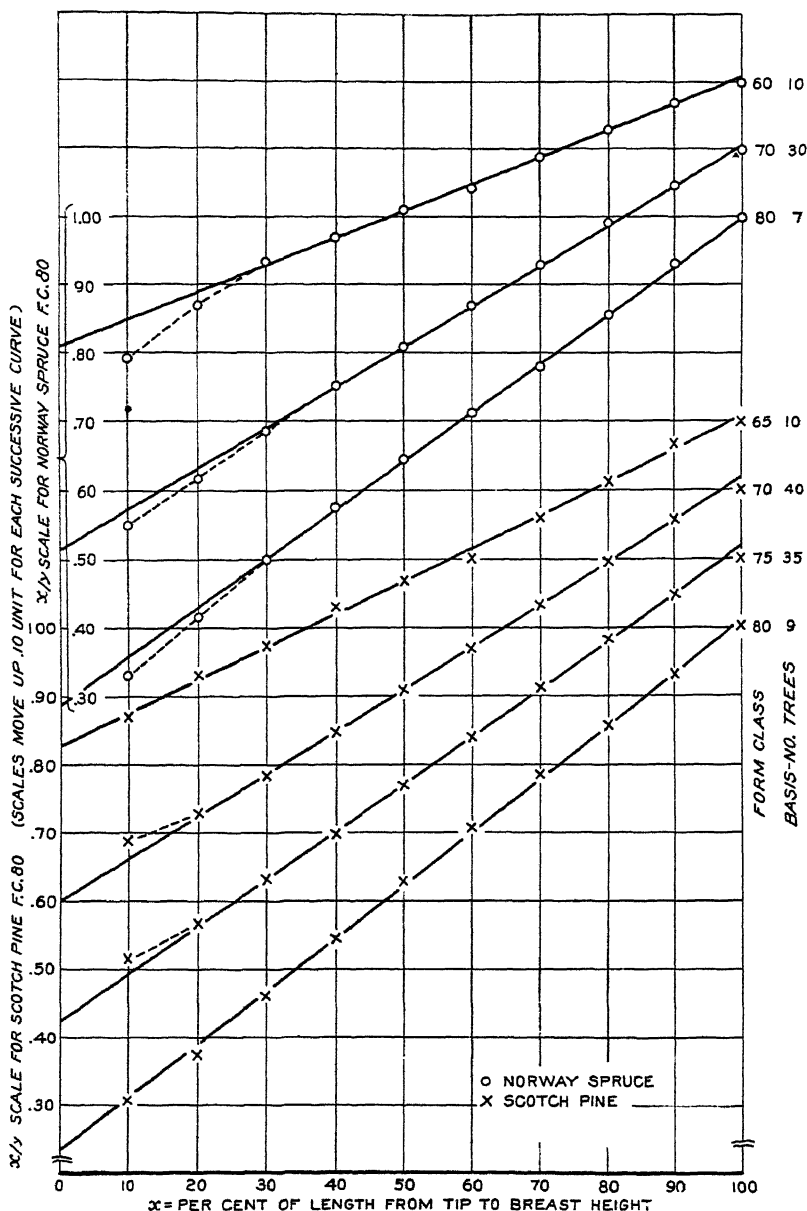


FIG. 3.—Plotting of  $x/y$  on  $x$  showing conformity to  $y = \frac{x}{a+bx}$ , from data on Norway spruce and Scotch pine as given by Tor Jonson

These charts reveal a striking similarity in the form of the various species with the exception of hemlock, and indicate, with this excep-

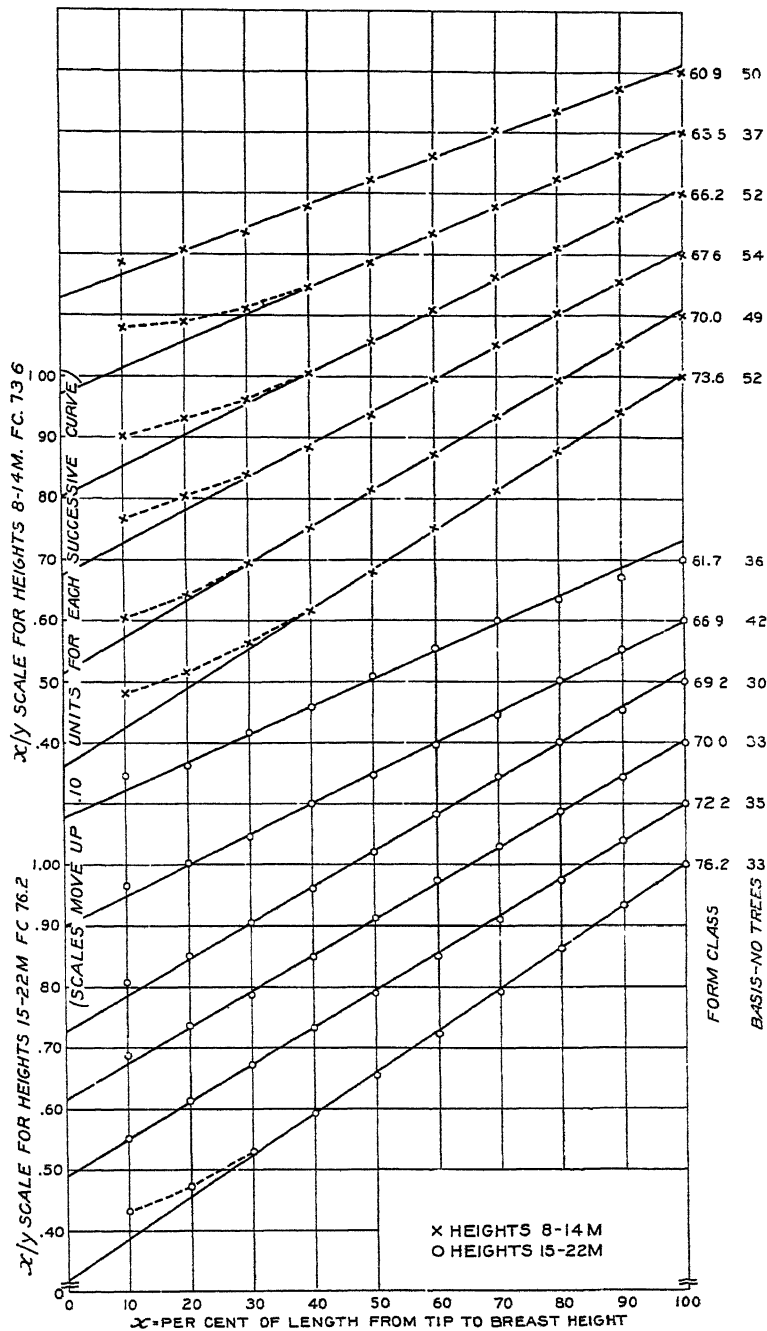


FIG. 4.—Plotting of  $x/y$  on  $x$  showing conformity to  $y = \frac{x}{a+bx}$ , from data on European larch as given by L. Mattsson

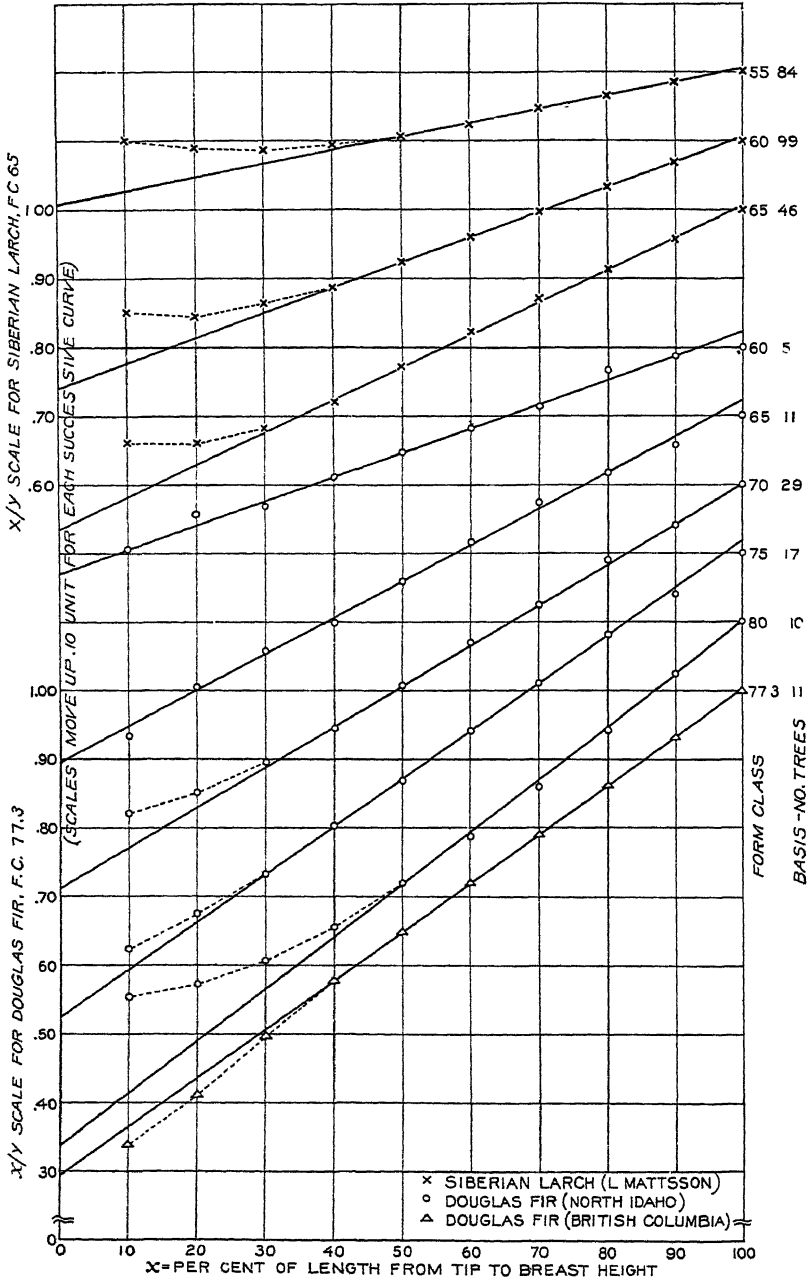


FIG. 5.—Plotting of  $x/y$  on  $x$  showing conformity to  $y = \frac{x}{a+bx}$ , from data on Siberian larch as given by L. Mattsson; Douglas fir, north Idaho; and Douglas fir, British Columbia

tion, a satisfactory conformity to the type equation  $y = \frac{x}{a+bx}$ . It will be noted, however, that in many of the species there is still a definite and constant tendency in some classes for the tops to fall below the values given by the formula, as indicated by the divergence

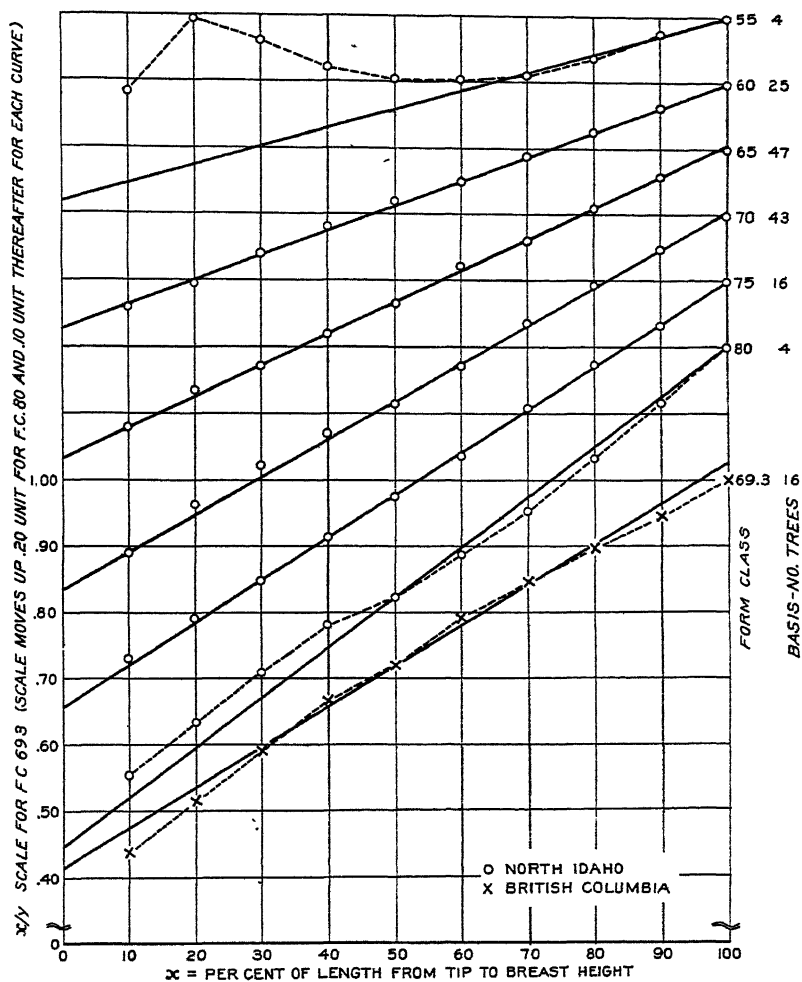


FIG. 6.—Plotting of  $x/y$  on  $x$  showing conformity to  $y = \frac{x}{a+bx}$ , from data on western white pine in north Idaho and British Columbia

upward from the straight lines of the points for the upper sections. This divergence in the tops varies with the different species, but seems to be fairly constant within the species. It is practically negligible in all the species studied except hemlock and balsam fir up to form class 65 but increases rather rapidly above form class 70. The nature of this variation will be discussed later.

## DIRECT COMPARISON OF SPECIES TAPERS

In order to bring all the material together for direct comparison the different series can be plotted on one chart by the method of

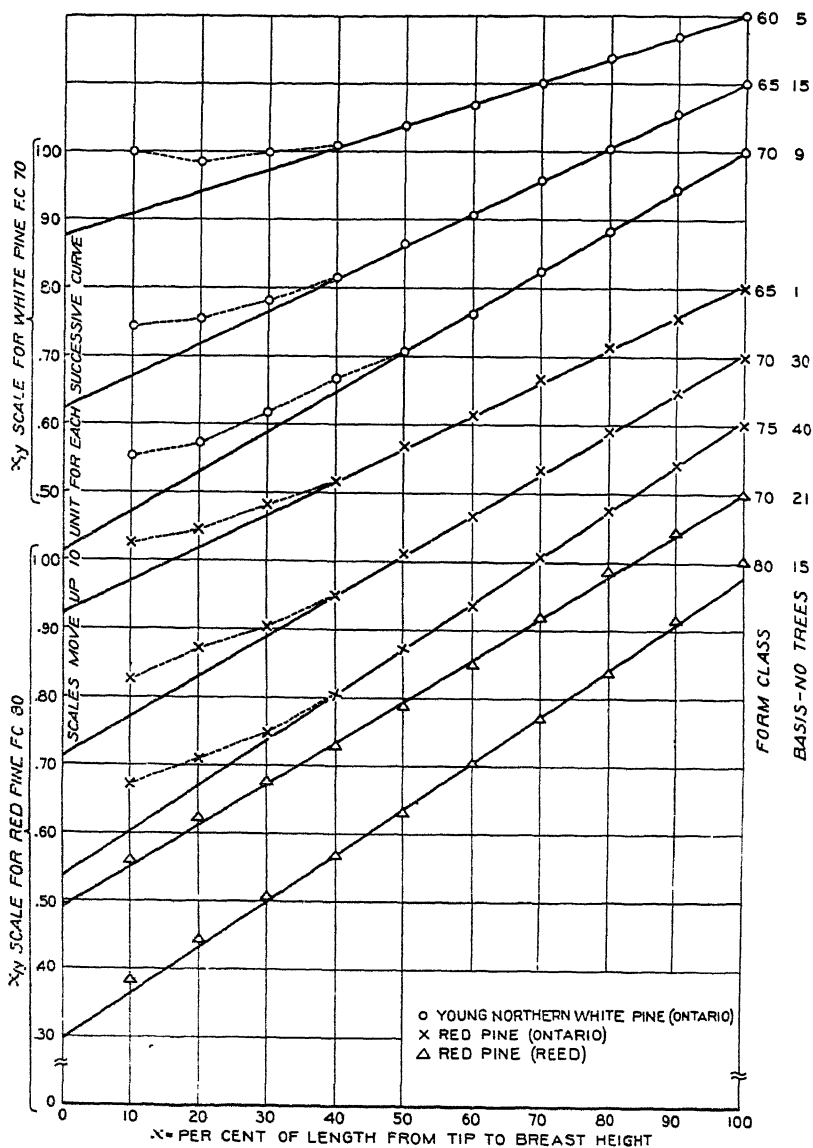


FIG. 7.—Plotting of  $x/y$  on  $x$  showing conformity to  $y = \frac{x}{a + bx}$ , from data on young northern white pine in Ontario, red pine in Ontario, and red pine as given by Reed

Figure 1. In this chart form classes are abscissae, values of  $y$  are ordinates and a separate curve is drawn for each value of  $x$ . The values for any series, therefore, appear as a set of points on the

vertical line corresponding to its true form class. In order to determine the proper form-class line on which to plot a series it is necessary first to be sure that root swell is correctly eliminated from the raw

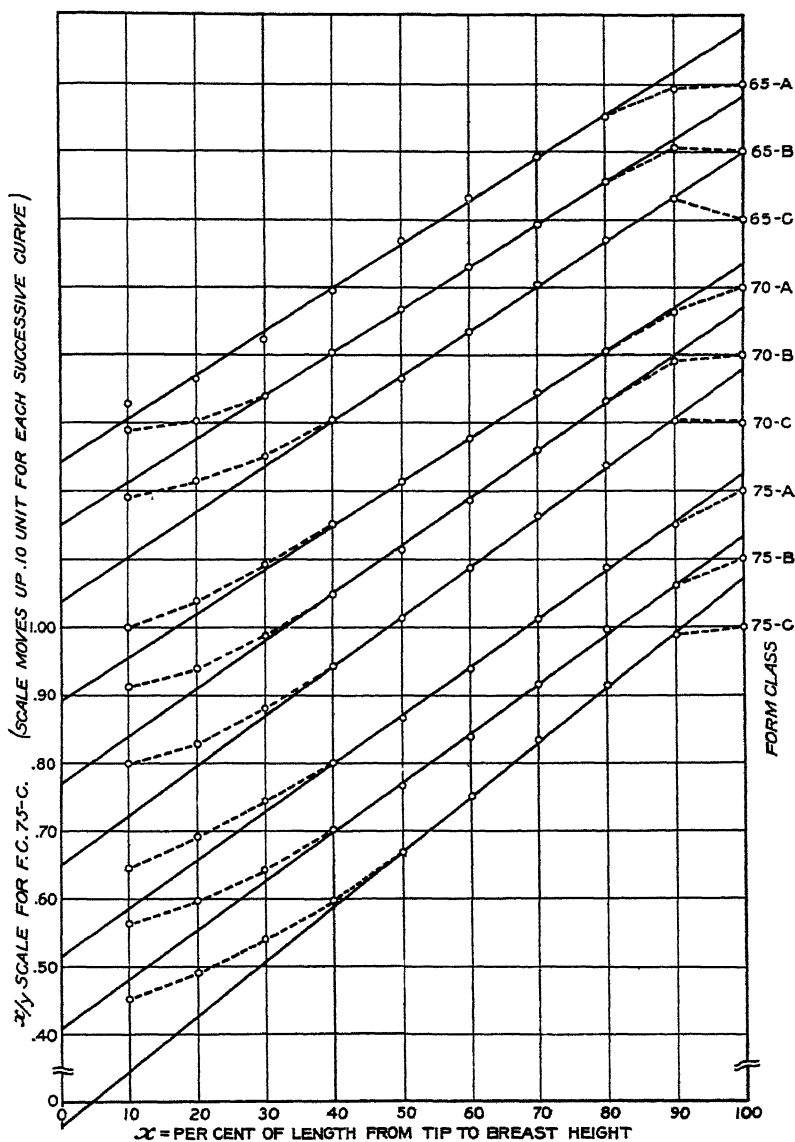


FIG. 8.—Plotting of  $x/y$  on  $x$  showing conformity to  $y = \frac{x}{a + bx}$ , from data on northern white pine in Canada, as given by W. G. Wright

averages, and, second; to examine the diameter at half height to find out whether it is erratic as compared to the measurements of adjacent sections and if so to bring it into line with them. These corrections are necessary because the form quotient derived from the

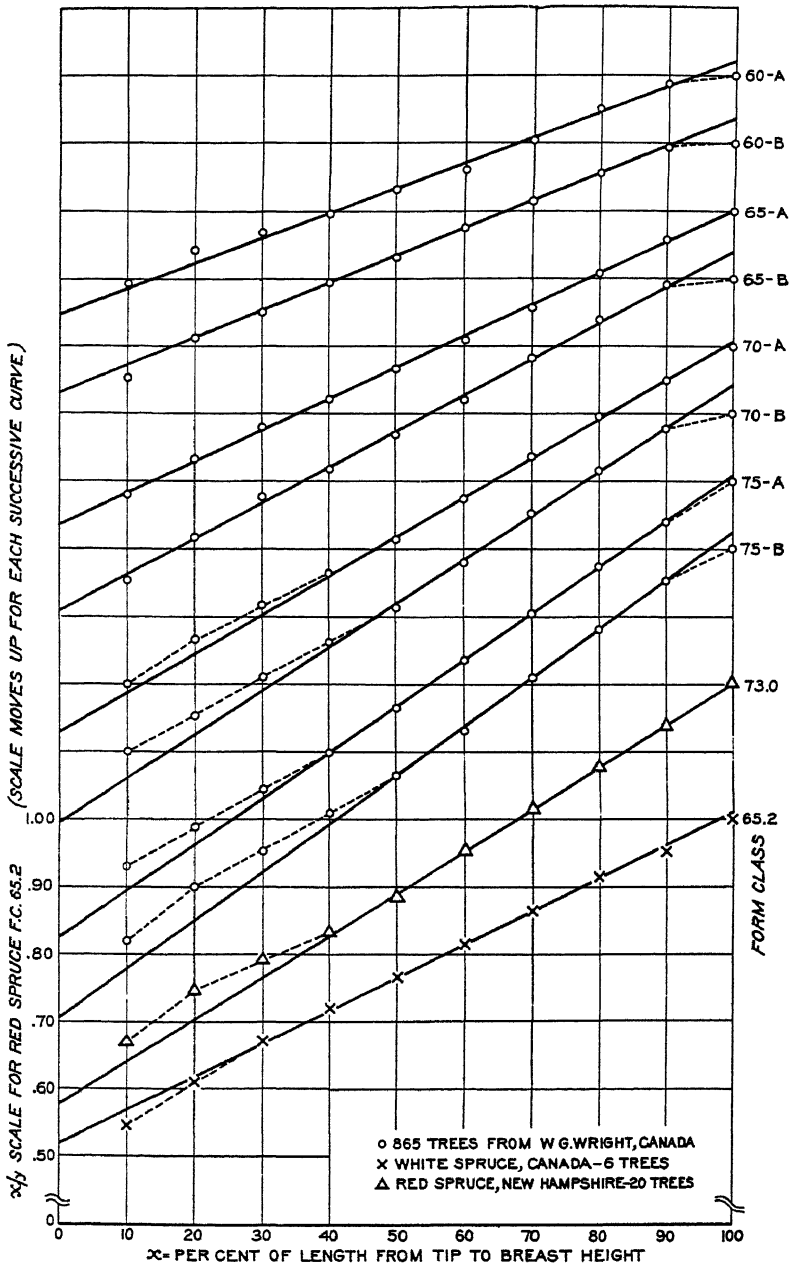


FIG. 9.—Plotting of  $x/y$  on  $x$  showing conformity to  $y = \frac{x}{a+bx}$ , from data on red, white, and black spruce

raw averages may not indicate the form curve corresponding most closely to the series in question if the diameter at breast height is distorted by root swell, or if through the chance errors of sampling

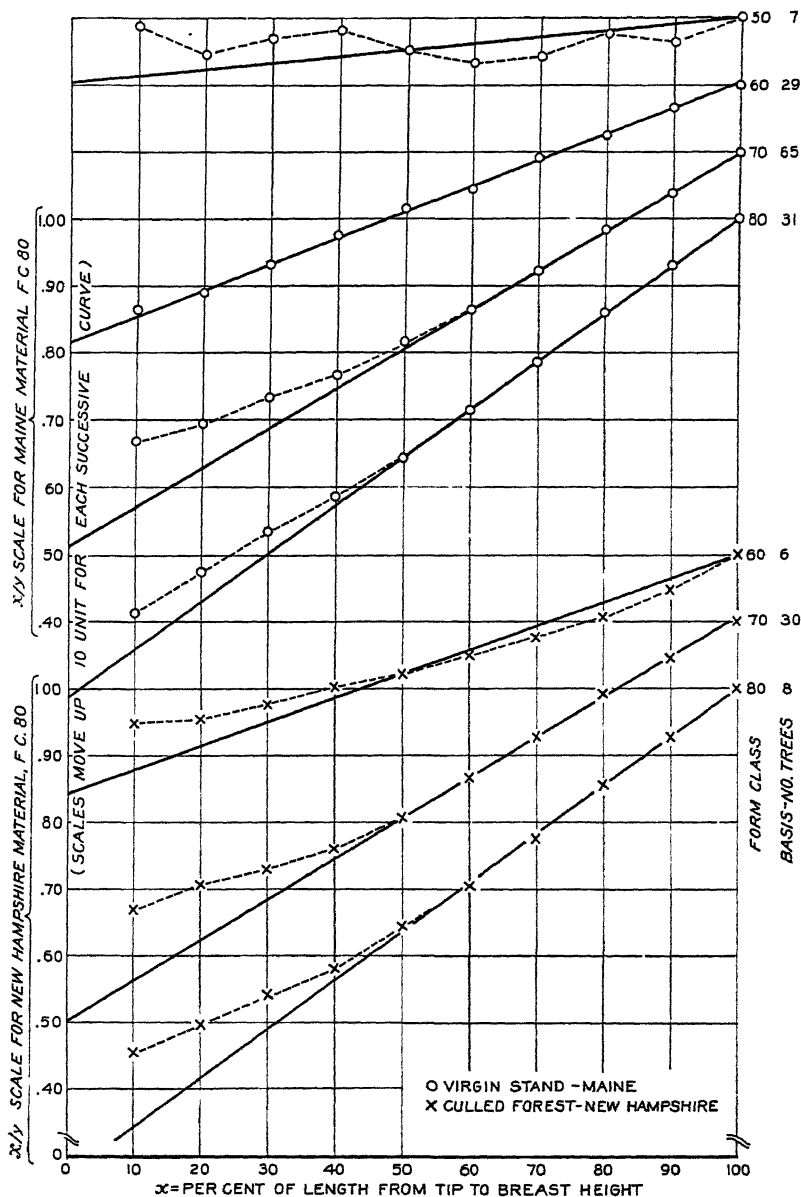


FIG. 10.—Plotting of  $z/y$  on  $x$  showing conformity to  $y = \frac{x}{a+bx}$ , from data on red spruce

either the diameter at breast height or the diameter at the mid-point is out of line with adjacent sections. Both of these corrections can be made easily and satisfactorily by the use of the straight line test charts of  $x/y$  on  $x$ .

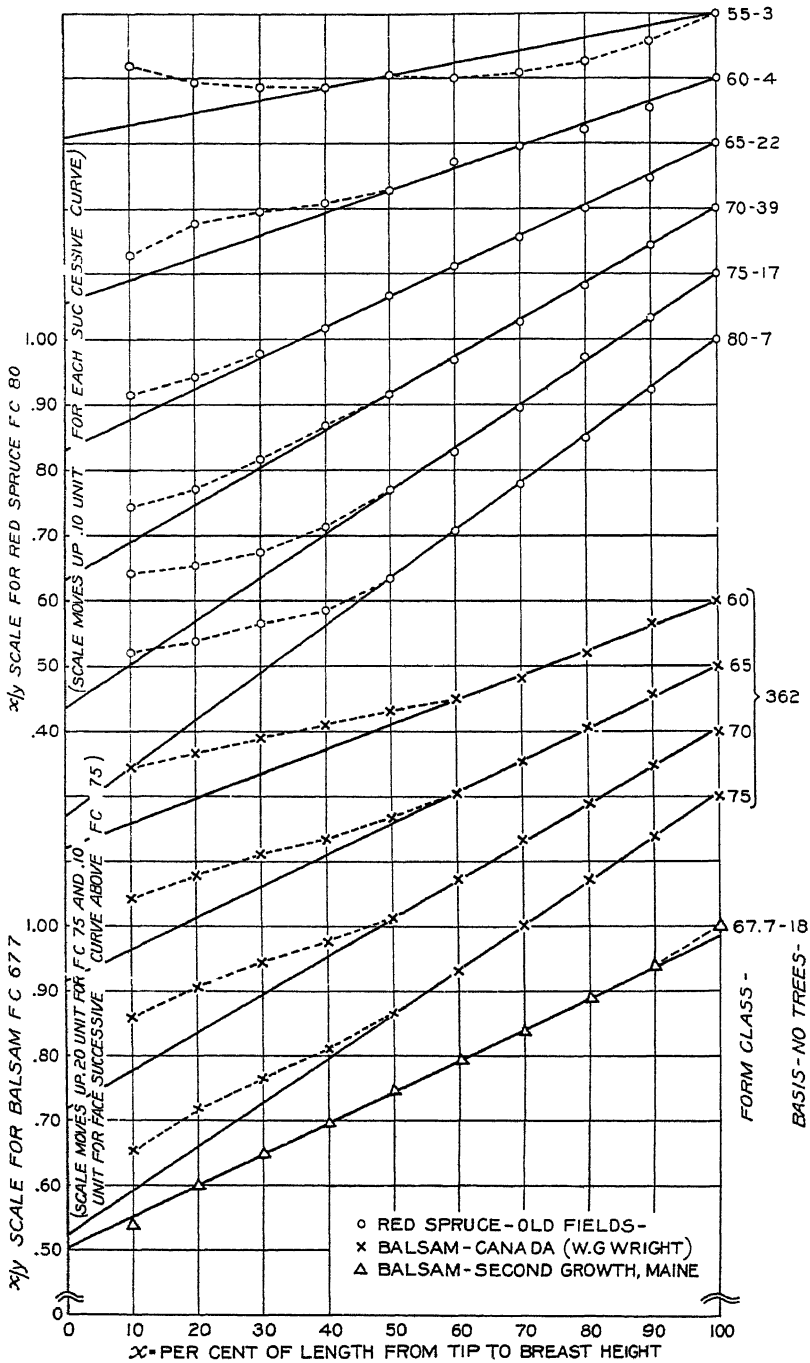


FIG. 11.—Plotting of  $x/y$  on  $x$  showing conformity to  $y = \frac{x}{a+bx}$ ; from data on old fields of red spruce; balsam fir in Canada, as given by W. G. Wright; and second-growth balsam fir in Maine

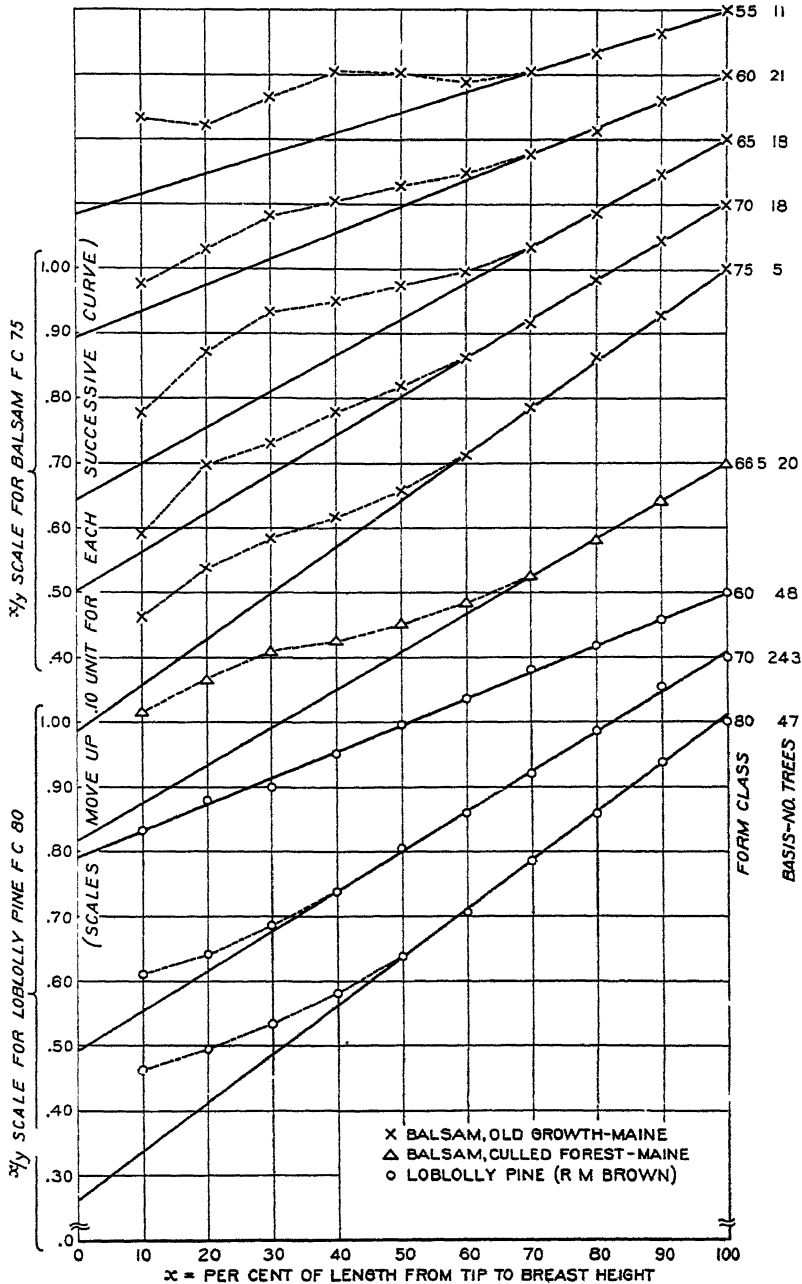


FIG. 12.—Plotting of  $x/y$  on  $x$  showing conformity to  $y = \frac{x}{a+bx}$ , from data on old-growth and culled forest balsam fir in Maine, and loblolly pine as given by R. M. Brown

In Figures 2 to 14 it will be noted that in many of the series, especially those in which no attempt was made to eliminate root swell in the preliminary compilation, the value for the breast-height point ( $x=100$ ) falls considerably below the straight line best fitting the intermediate sections of the tree. This deviation is the result

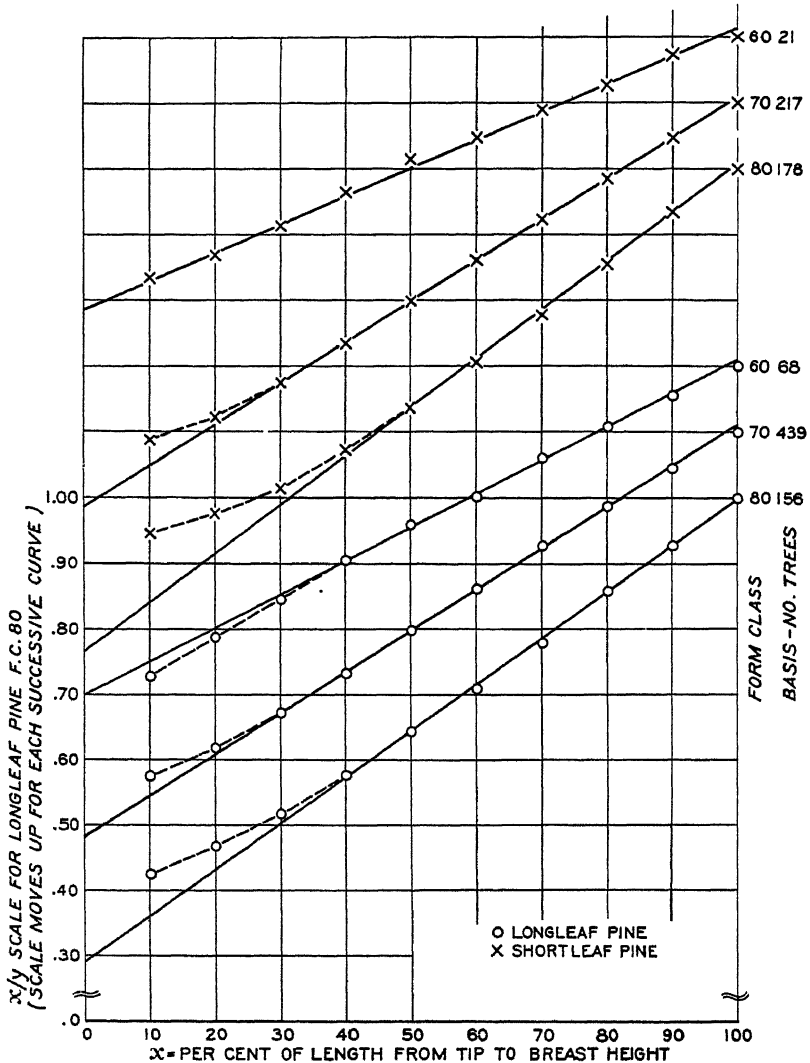


FIG. 13.—Plotting of  $x/y$  on  $x$  showing conformity to  $y = \frac{x}{a+bx}$ , from data on long-leaf and short-leaf pine as given by R. M. Brown

of the distortion of the normal curve by root swell, and so the true normal diameter at breast height can be calculated from the value of  $x/y$  read from the intersection of the best fitting straight line and its breast height abscissa. Thus, in Figure 3, Scotch pine F. C. 80, the straight line best fitting the plotted points in the intermediate

sections crosses the breast height abscissa at 1.012. The diameter to be used as the base for the true normal form quotient is, therefore,  $\frac{100}{1.012} = 98.8$ . In a similar manner an examination of the figure shows that the value at half height ( $x=50$ ) falls slightly above the line giving best fit to the series as a whole, indicating that this average may be considered somewhat erratic. The middle diameter

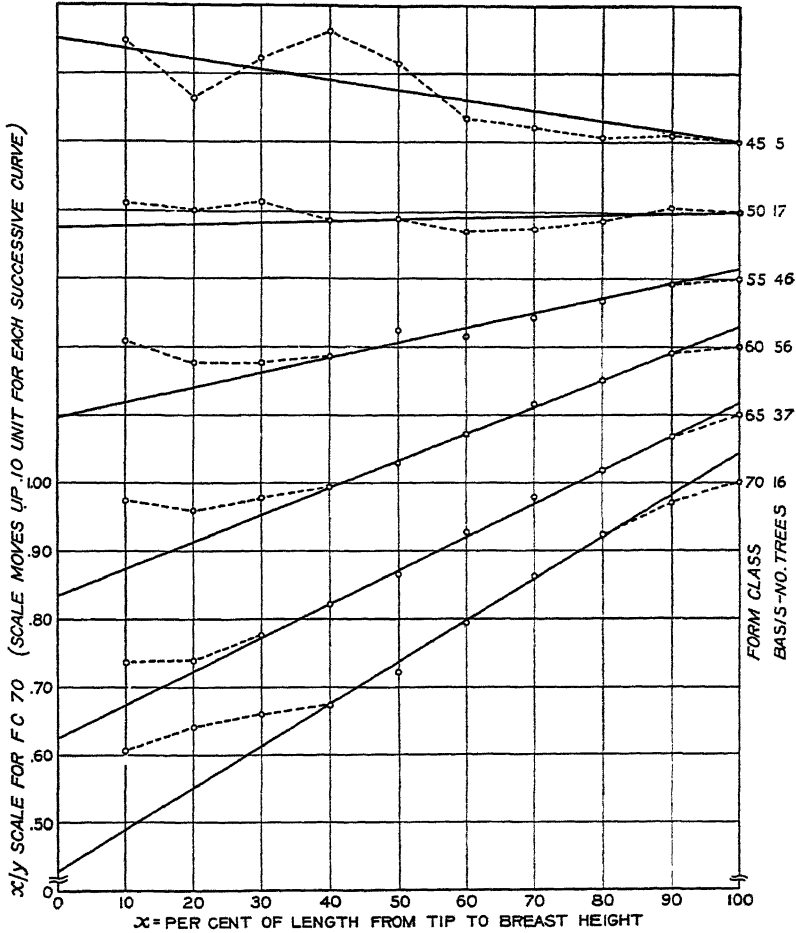


FIG. 14.—Plotting of  $x/y$  on  $x$  showing conformity to  $y = \frac{x}{a+bx}$ , from data on gray birch as given by R. C. Hawley

to be used in calculating the true normal form quotient is determined from the intersection of the best fitting straight line with the abscissa for the midpoint. Thus,  $x/y = .624$  and  $\frac{50}{.624} = 80.1$ , the "normal" diameter at half height. The form class line upon which this series should be plotted in our comparative chart is, therefore,  $\frac{80.1}{98.8} \times 100 = 81.1$  instead of 79.4 as indicated by the raw material.

This new value of the form quotient is more reliable than that obtained from the raw material because it is based on the fit of the entire stem rather than upon the arbitrary field measurement of the two points in question. This principle can be applied to those series in which root swell was eliminated graphically before averaging as well as to those in which the averaging was done directly from the

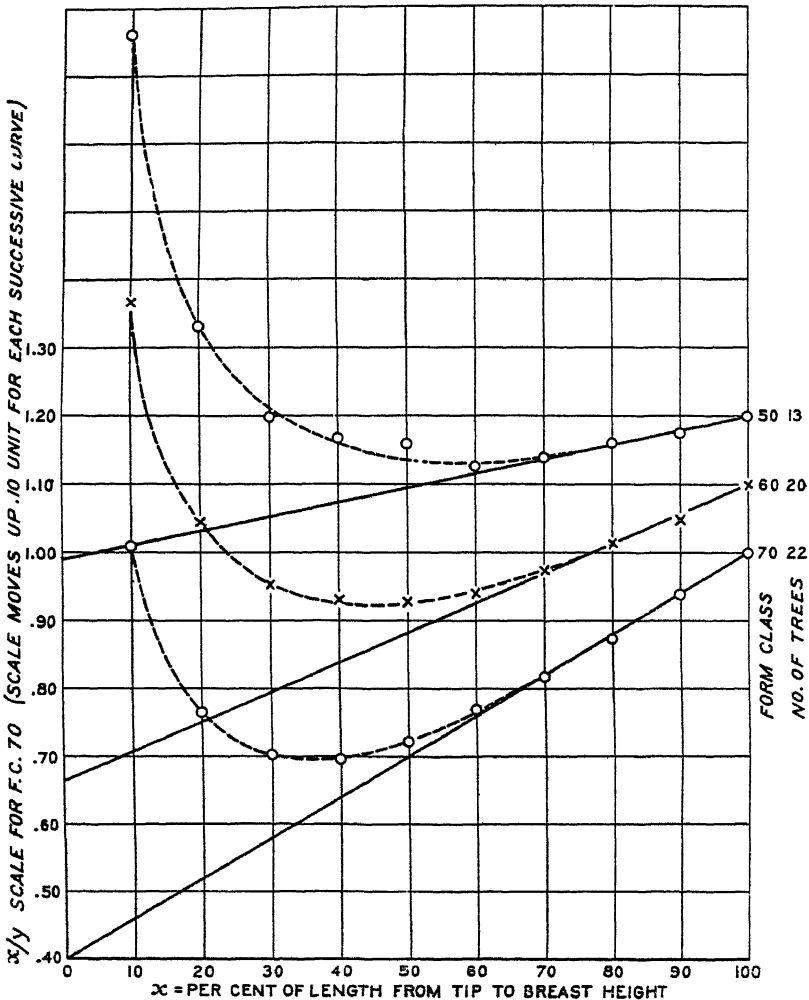


FIG. 15.—Plotting of  $x/y$  on  $x$  showing conformity to  $y = \frac{x}{a+bx}$ , from data on eastern hemlock

tree measurements, and serves as a check upon the graphic method. The straight-line test will reveal in the average any consistent tendency to exaggerate, or failure to make sufficient allowance for root swell in the plotting of the individual trees. In applying this principle to series where the divergence of the material from a straight line is marked, as with balsam, the straight line used to determine the normal form class is that best fitting the lower portions

of the stem, because only in this way can a direct comparison of the amount of divergence of the tops from the formula be obtained for the different species.

After determining the true normal form quotient for each series by the method just described, all the material with the exception of hemlock, which requires special treatment, has been brought together for direct comparison on a single chart. Whenever the diameter at breast height had been modified to eliminate root swell the remain-

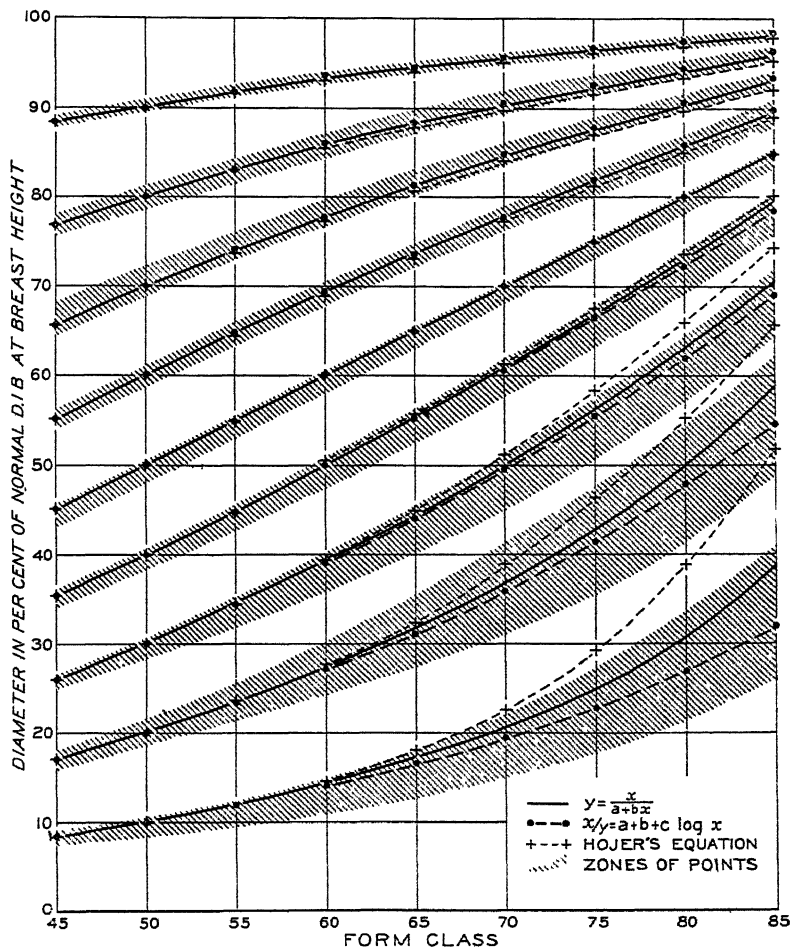


FIG. 16.—Range of percentile tapers compared with formulae

ing percentile tapers were recomputed with the true "normal" diameter as a base in order to compare them properly with other series. The data for this comparison are brought together in Table 4. Because of the large number of points grouped closely together it is difficult to identify the individual series in the plotting; but the points for the different sections fall into well-defined bands. Figure 16 shows the approximate upper and lower limits of the band of

points at each section together with the curves given by Höjer's equation, by the simple form  $y = \frac{x}{a+bx}$ , and by a modification of the latter to be discussed presently.

TABLE 4.—Normal form quotients and adjusted percentile tapers  
WESTERN YELLOW PINE

Form class	Normal form quotient	Percentage of length from tip to breast height= $x$									Basis number of trees
		90	80	70	60	50	40	30	20	10	
		Adjusted percentile tapers									
45-----	46.0	89.7	76.1	68.9	55.7	46.6	36.2	26.0	17.7	10.0	3
55-----	55.6	92.2	83.3	74.3	63.5	54.8	45.8	34.5	24.2	12.7	6
60-----	60.2	93.8	85.5	78.2	69.8	59.9	50.0	37.8	27.3	15.1	17
65-----	66.1	93.5	88.6	82.6	75.5	65.7	56.5	44.6	31.0	18.8	29
70-----	70.2	95.2	90.5	85.1	78.7	70.6	60.9	50.2	36.5	21.2	55
75-----	75.0	96.5	92.4	87.7	81.8	75.4	66.7	55.6	40.9	23.3	70
80-----	79.1	96.7	93.9	90.7	85.2	79.1	70.3	59.4	44.4	25.7	18
85-----	82.6	97.5	95.1	92.6	87.5	83.5	74.9	61.2	45.4	33.1	3

NORWAY SPRUCE (JONSON-1910)

60-----	62.2	93.6	86.7	79.3	71.5	62.1	52.3	41.2	30.0	17.0	10
70-----	70.7	95.7	90.5	84.9	78.4	70.7	61.7	51.3	38.8	22.4	30
80-----	77.6	96.6	93.3	89.4	83.9	77.3	69.6	60.0	48.1	30.3	7

SCOTCH PINE (JONSON-1911)

65-----	65.6	93.9	88.5	82.2	75.7	65.7	55.4	44.9	32.0	17.7	10
70-----	71.9	95.8	90.9	85.7	79.4	71.9	62.9	52.4	38.6	20.8	40
75-----	75.9	96.7	92.4	87.8	82.6	76.0	68.1	57.3	43.5	24.5	35
80-----	81.1	97.8	94.3	90.3	85.6	80.4	74.1	65.7	54.0	33.0	9

EUROPEAN LARCH—HEIGHTS 8 TO 14 METERS (MATTSSON-1917)

60.9-----	61.8	94.0	86.6	78.8	70.8	61.9	52.3	41.5	28.8	14.8	50
63.5-----	63.9	94.0	87.4	80.5	72.7	64.0	54.0	42.4	29.2	14.8	37
66.2-----	66.8	94.9	88.9	82.2	75.1	66.9	57.4	45.9	32.0	16.8	52
67.6-----	67.9	95.0	89.2	83.0	76.1	68.2	58.8	47.2	33.4	17.8	54
70.0-----	70.8	95.8	90.6	84.9	78.5	70.9	62.0	51.1	37.2	20.1	49
73.6-----	73.4	96.2	91.5	86.6	80.2	74.0	65.1	53.7	38.7	20.8	52

EUROPEAN LARCH—HEIGHTS 15 TO 22 METERS (MATTSSON-1917)

61.7-----	64.1	95.8	88.1	80.4	72.5	63.8	54.4	43.2	31.1	15.9	36
66.9-----	66.7	94.4	88.5	82.4	75.3	66.9	57.1	46.2	33.2	17.6	42
69.2-----	70.5	96.2	90.6	84.4	78.1	70.6	61.6	50.6	36.9	20.1	30
70.0-----	70.5	95.7	90.5	84.7	77.9	70.5	61.8	51.4	37.4	20.5	33
72.2-----	71.9	95.8	91.5	86.3	79.9	72.2	63.2	52.3	38.8	22.1	35
76.2-----	75.6	96.5	92.7	88.3	82.9	76.2	67.6	56.6	42.4	23.1	33

SIBERIAN LARCH (MATTSSON-1917)

55-----	55.4	91.9	83.1	74.4	65.2	55.4	44.9	34.0	22.6	11.2	84
60-----	61.1	93.4	86.3	78.5	70.2	61.1	51.1	39.5	27.0	13.4	99
65-----	65.4	94.7	88.2	81.2	73.5	65.4	56.1	44.3	30.5	15.2	46

TABLE 4.—Normal form quotients and adjusted percentile tapers—Continued  
WESTERN WHITE PINE (BEHRE-1923 AND U. S. F. S.-1910-1913)

Form class	Normal form quot- ient	Percentage of length from tip to breast height= $x$									Basis num- ber of trees
		90	80	70	60	50	40	30	20	10	
		Adjusted percentile tapers									
55-----	58.2	92.4	85.2	76.8	66.4	55.2	43.3	31.1	20.1	11.3	4
60-----	61.4	93.4	86.2	78.8	70.5	60.8	51.1	40.4	28.7	15.1	25
65-----	65.7	94.3	88.4	81.4	73.4	65.4	55.5	44.7	31.4	17.3	47
70-----	69.7	95.3	89.9	84.0	78.0	70.0	59.9	48.3	35.6	20.4	43
75-----	73.7	96.2	91.5	86.4	81.2	74.0	65.3	54.5	40.7	23.2	16
80-----	80.2	98.0	95.8	92.8	87.3	80.2	68.8	58.7	46.1	28.1	4

## WESTERN WHITE PINE (CLAUGHTON-WALLIN AND McVICKER-1920)

Average----	71.2	97.6	91.7	84.9	77.8	71.2	61.8	52.2	39.8	23.3	16
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## DOUGLAS FIR (U. S. F. S.-1910-1913)

60-----	60.4	93.2	84.6	78.4	69.7	60.3	50.5	40.0	27.0	14.5	5
65-----	67.3	96.0	89.0	82.0	75.0	67.3	58.5	46.7	33.9	19.1	11
70-----	70.5	95.6	89.8	84.7	77.9	70.5	61.8	50.2	36.2	19.2	29
75-----	75.9	97.8	92.6	87.8	82.4	76.2	67.6	57.4	43.0	24.1	17
80-----	80.6	97.5	95.0	92.1	87.3	80.6	72.0	59.2	42.2	22.0	10

## DOUGLAS FIR (CLAUGHTON-WALLIN AND McVICKER-1920)

Average----	77.4	96.8	93.0	88.6	83.6	77.4	69.5	60.6	48.7	29.6	11
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## NORWAY PINE (CLAUGHTON-WALLIN AND McVICKER-1920)

65-----	65.8	94.7	88.2	81.3	74.3	65.5	56.1	44.2	31.2	16.1	11
70-----	70.8	95.5	90.4	84.7	78.6	70.7	61.8	49.9	35.2	19.1	30
75-----	75.0	96.3	92.2	87.3	82.1	75.0	66.5	55.1	39.4	21.4	40

## NORWAY PINE (REED-1924)

70-----	71.8	95.4	90.5	85.6	80.0	72.6	63.6	51.8	38.2	21.7	21
80-----	77.0	96.3	93.4	88.7	83.3	77.2	68.9	58.1	44.6	25.2	15

## YOUNG NORTHERN WHITE PINE (CLAUGHTON-WALLIN AND McVICKER-1920)

60-----	59.7	93.0	85.5	77.8	69.0	59.7	49.4	37.6	25.5	12.5	5
65-----	65.7	94.2	88.4	81.7	74.3	65.5	55.9	44.0	30.5	15.5	15
70-----	70.8	95.2	90.6	84.9	78.6	70.8	60.0	48.5	34.9	18.0	9

## OLD NORTHERN WHITE PINE (WRIGHT-1923)

65-A-----	71.0	97.9	90.7	84.7	78.1	70.3	62.2	52.2	38.3	20.4	342
65-B-----	70.6	96.8	90.6	84.8	78.2	70.5	61.8	50.8	36.0	18.4	
65-C-----	71.5	96.0	90.7	85.1	79.1	71.6	62.3	50.7	35.8	18.6	
70-A-----	72.5	96.5	91.1	85.7	79.7	72.5	63.5	52.4	38.3	20.7	
70-B-----	74.2	97.1	91.8	87.0	81.6	74.9	66.1	54.6	39.6	20.8	
70-C-----	75.4	96.8	92.2	87.6	82.2	75.7	67.0	55.7	40.9	21.6	
75-A-----	78.4	97.0	92.5	88.2	83.2	76.9	68.1	56.4	41.5	22.9	
75-B-----	76.8	96.6	92.2	88.3	83.8	77.5	68.5	56.8	41.4	22.2	
75-C-----	80.2	97.6	93.8	89.9	85.8	80.5	71.9	59.2	43.6	23.6	

TABLE 4.—Normal form quotients and adjusted percentile tapers—Continued  
WHITE, RED, AND BLACK SPRUCE (WRIGHT—1923)

Form class	Normal form quot- ient	Percentage of length from tip to breast height= $x$									Basis num- ber of trees
		90	80	70	60	50	40	30	20	10	
		Adjusted percentile tapers									
60-A-----	61.1	93.1	85.9	79.0	71.2	61.3	51.1	39.9	27.6	14.7	865
60-B-----	62.1	93.7	86.7	79.1	71.0	62.2	52.1	41.3	29.0	15.8	
65-A-----	65.4	94.6	88.7	82.2	74.5	65.5	55.7	44.4	31.8	17.3	
65-B-----	67.1	94.6	88.7	82.5	76.1	67.6	58.1	46.7	33.7	18.7	
70-A-----	70.1	95.4	90.0	84.4	77.9	70.5	60.6	48.8	35.4	20.1	
70-B-----	72.3	95.6	90.7	85.4	79.9	72.8	62.6	50.9	37.4	20.8	
75-A-----	75.4	96.4	92.2	87.7	82.3	75.8	67.2	55.4	41.2	23.4	
75-B-----	76.8	96.8	92.9	88.5	83.8	76.8	67.1	55.3	41.0	24.0	

WHITE SPRUCE (CLAUGHTON-WALLIN AND McVICKER—1920)

Average....	65.9	95.3	88.4	81.5	74.1	65.9	56.1	45.2	33.1	18.4	6
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VIRGIN RED SPRUCE (U. S. F. S.—1902)

50-----	52.4	93.2	81.7	74.1	64.1	52.4	40.6	30.9	21.1	10.1	7
60-----	62.0	93.6	86.8	79.0	71.2	61.7	51.7	41.1	29.1	15.1	29
70-----	70.9	95.8	90.4	84.8	78.2	69.8	59.9	47.2	33.6	17.5	65
80-----	77.8	96.6	93.0	89.0	84.0	77.6	68.1	56.0	42.0	24.2	31

RED SPRUCE-CULLED FOREST (U. S. F. S.—1903)

60-----	60.8	94.8	88.2	79.8	70.6	60.8	49.9	38.6	26.5	13.3	6
70-----	71.4	96.0	90.6	85.2	79.0	71.2	60.8	47.9	33.2	17.7	30
80-----	78.5	96.8	93.4	90.1	85.0	77.3	68.6	55.1	40.0	21.9	8

SECOND-GROWTH RED SPRUCE (U. S. F. S.—1903)

Average....	72.6	95.8	91.2	86.0	79.8	73.0	63.1	50.7	36.7	21.3	20
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OLD-FIELD RED SPRUCE (U. S. F. S.—1903 AND MEYER—1924)

55-----	55.3	94.2	86.5	76.9	66.7	55.3	45.3	33.9	22.5	10.9	3
60-----	60.3	94.3	86.8	78.1	68.8	60.3	49.5	37.7	25.8	13.7	4
65-----	65.2	95.1	88.8	81.8	74.0	65.2	55.9	44.2	31.2	16.2	22
70-----	69.9	95.3	90.7	84.8	78.2	69.9	59.9	48.5	35.0	18.4	39
75-----	74.9	96.4	91.7	87.9	82.4	74.9	65.2	52.3	36.0	18.5	17
80-----	78.9	97.5	94.1	90.1	84.9	78.9	68.5	53.2	37.2	19.2	7

BALSAM FIR (WRIGHT—1923)

60-----	61.6	93.3	86.9	79.4	70.5	60.0	49.3	38.0	26.1	13.4	362
65-----	65.9	94.0	88.2	82.0	74.6	65.0	54.4	42.2	29.5	15.5	
70-----	70.6	95.8	90.5	84.7	78.1	70.6	59.5	47.0	33.3	18.0	
75-----	75.5	96.5	92.2	87.9	82.4	75.5	65.6	53.4	39.0	22.2	

SECOND-GROWTH BALSAM FIR (MEYER—1923)

Average....	66.2	94.5	88.7	82.3	74.8	66.7	56.6	45.5	32.8	18.3	18
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TABLE 4.—Normal form quotients and adjusted percentile tapers—Continued

## BALSAM FIR—CULLED FOREST (BEHRE—1924)

Form class	Normal form quotient	Percentage of length from tip to breast height= $x$									Basis number of trees
		90	80	70	60	50	40	30	20	10	
		Adjusted percentile tapers									
Average----	70.7	95.4	90.9	84.9	76.6	66.5	55.2	42.2	30.0	16.2	20

## BALSAM FIR—OLD GROWTH (BEHRE—1924)

55.....	59.5	93.6	85.6	77.4	67.7	55.5	44.1	34.7	24.3	12.0	11
60.....	62.9	93.9	87.4	79.8	70.8	60.3	49.6	38.4	27.4	14.8	21
65.....	69.2	95.2	90.4	84.1	75.4	64.6	53.2	40.8	29.8	17.3	18
70.....	71.4	95.6	90.8	86.0	78.9	69.7	59.0	47.6	33.6	20.5	18
75.....	77.6	96.8	92.7	89.0	84.2	75.9	64.7	51.4	37.1	21.6	5

## LONGLEAF PINE (BROWN—1924)

60.....	66.9	95.2	88.9	82.3	75.7	64.5	57.2	46.9	34.3	19.1	68
70.....	72.6	96.4	91.4	85.7	80.0	72.6	64.3	53.2	39.0	21.3	439
80.....	77.9	97.0	93.3	90.0	84.8	77.9	69.5	58.0	42.8	23.5	156

## SHORTLEAF PINE (BROWN—1924)

60.....	63.5	94.2	87.7	80.0	71.8	62.3	53.5	42.9	30.5	16.1	21
70.....	72.4	95.9	91.3	86.0	79.6	72.6	63.8	52.8	38.6	20.7	217
80.....	79.2	97.4	94.5	90.5	85.6	79.2	70.5	58.6	42.2	22.6	178

## LOBLOLLY PINE (BROWN—1924)

60.....	62.7	94.0	87.0	79.5	71.7	62.7	53.2	42.8	29.4	15.8	48
70.....	72.0	95.2	91.2	86.1	79.7	71.7	63.3	51.7	37.2	19.7	243
80.....	79.2	97.2	94.2	90.3	85.9	79.2	69.6	56.9	41.0	21.9	47

## GRAY BIRCH (HAWLEY—1923)

45.....	46.5	89.2	79.7	68.6	58.1	44.8	34.4	26.7	18.8	8.7	5
50.....	50.6	89.7	81.3	72.1	61.9	50.6	40.5	29.6	20.0	9.9	17
55.....	56.0	91.8	83.7	75.4	66.4	54.9	45.8	34.7	23.1	11.2	46
60.....	61.9	93.5	86.5	78.7	70.8	62.0	51.9	39.8	27.1	13.3	56
65.....	66.0	94.7	88.5	81.1	73.8	66.5	56.3	45.1	31.8	16.0	37
70.....	70.8	96.7	90.2	84.8	78.8	72.1	61.9	47.4	32.6	17.2	16

It should be borne in mind that the spread of the bands of points for the sections  $x=10$  and  $x=20$  would be materially reduced if series whose tops depart markedly from the equation  $y = \frac{x^2}{a+bx}$ , such as those for balsam fir, were plotted on a lower form-class line, for this would balance small diameters in the tops against larger diameters in the lower sections. This procedure, however, would increase the spread of the bands for the lower sections of the stem and make direct comparison of differences in the tops impossible. The spread of points indicated is materially lessened, especially in the zones for  $x=20$  and  $x=30$ , when the data for balsam fir in Figure 12 are omitted. The spread of the points in these bands is indicative of the variations

between the different species. If the bands are narrow it means that there is little difference in the tapers of the different species, and that a single taper series may be used for all, but if the bands are broad and the points widely scattered within them considerable variation between the species is indicated, and the use of a generalized taper series for all the species becomes of doubtful accuracy.

#### CONSTANTS AND ABSOLUTE FORM FACTORS

To use the formula  $y = \frac{x}{a + bx}$  as the basis for generalized taper tables requires the evaluation of the constants  $a$  and  $b$ . These constants vary with the form quotient and may readily be calculated by substituting the two pairs of values for the variables which are fixed for any given form class by definition of form quotient. Thus, for form class 75 we have  $x = .50$ ,  $y = .75$  and  $x = 1.00$ ,  $y = 1.00$ .

Therefore,

$$.75 = \frac{.50}{a + .50b} \quad .667 = a + .50b$$

$$1.00 = \frac{1.00}{a + 1.00b} \quad 1.000 = a + 1.00b$$

Subtracting

$$.333 = + .50b$$

$$b = .667$$

Substituting

$$1.00 = a + .667$$

$$a = .333$$

Table 5 gives the constants for form classes 40 to 90 at intervals of five units. Since in all instances  $a + b = 1$ , it is only necessary to know one constant to solve the equation. In Figure 17 the constants are presented graphically in order to permit rapid approximation of the constants for any desired form quotient without calculation. With these constants percentile tapers for the entire stem can be computed, as presented in Table 5.

TABLE 5.—Generalized percentile taper series; based on  $y = \frac{x}{a + bx}$

Form class	Percentage of length from tip to breast height									Constants		Abso- lute form factors
	90	80	70	60	50	40	30	20	10	a	b	
	Percentage of "normal" d. i. b. <sup>a</sup> at breast height											
40-----	85.71	72.73	60.87	50.00	40.00	30.77	22.22	14.29	6.90	1.5000	—0.5000	0.2688
45-----	88.03	76.60	65.62	55.10	45.00	35.29	25.96	16.98	8.33	1.2222	-.2222	.3008
50-----	90.00	80.00	70.00	60.00	50.00	40.00	30.00	20.00	10.00	1.0000	.0000	.3333
55-----	91.67	83.02	74.03	64.70	55.00	44.90	34.38	23.40	11.96	.8182	.1818	.3661
60-----	93.10	85.72	77.78	69.23	60.00	50.00	39.13	27.27	14.29	.6667	.3333	.4081
65-----	94.36	88.13	81.24	73.58	65.00	55.32	44.31	31.71	17.11	.5385	.4615	.4414
70-----	95.45	90.32	84.74	77.78	70.00	60.86	50.00	36.84	20.59	.4286	.5714	.4827
75-----	96.43	92.30	87.50	81.82	75.00	66.67	56.25	42.85	25.00	.3333	.6667	.5281
80-----	97.30	94.12	90.32	85.71	80.00	72.73	63.16	50.00	30.77	.2500	.7500	.5792
85-----	98.08	95.77	92.96	89.47	85.00	79.07	70.82	58.62	38.64	.1765	.8235	.6385
90-----	98.78	97.30	95.46	93.11	90.00	85.71	79.41	69.23	50.00	.1111	.8889	.7111

<sup>a</sup> D. i. b. = diameter inside bark.

The absolute form factor with the tree volume computed in 10 sections by the Smalian formula is expressed by the formula—

$$\frac{D^2}{2} + d_1^2 + d_2^2 + d_3^2 + \dots + d_9^2$$

$$10D^2$$

in which  $D, d_1, d_2, d_3, \dots, d_9$  are the diameters at breast height and at each succeeding tenth of the stem above breast height. This

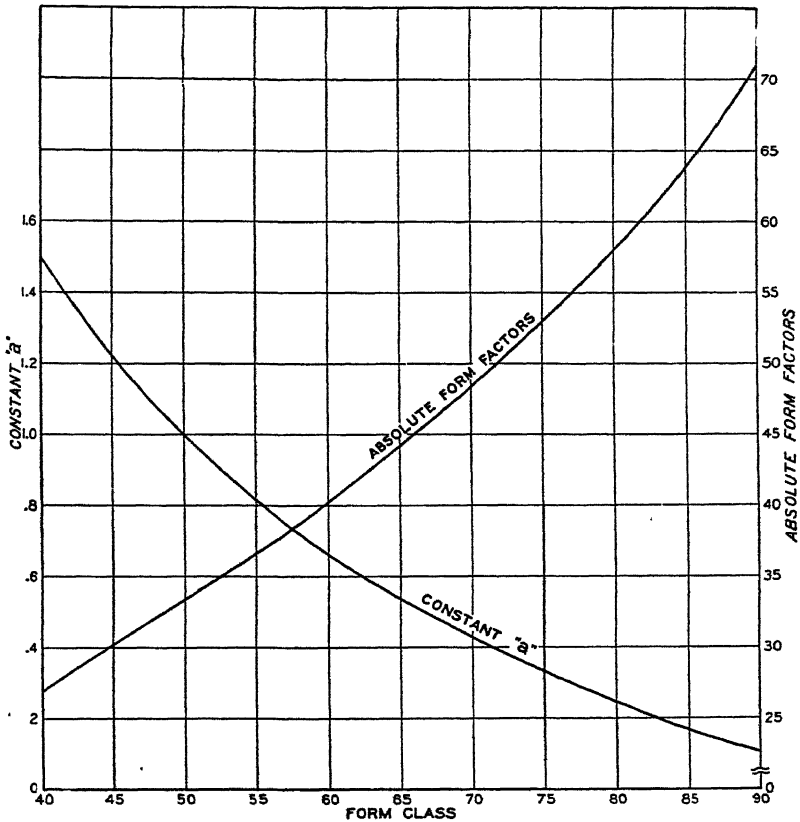


FIG. 17.—Absolute form factors and constants for  $y = \frac{x}{a+bx}$ . Constant  $b = 1-a$

method is in conformity with the conventional method of computing cubic contents, and was used by Jonson in compiling his volume tables. The Smalian formula, however, assumes the stem to be paraboloidal in form, and hence introduces a slight error which increases as the length of the sections into which the stem is divided increases. Absolute form factors corresponding exactly to the volumes generated by the stem curve, as expressed by the equation  $y = \frac{x}{a+bx}$  revolving about the axis, can be computed with the aid of integral calculus.

The volume of a solid of revolution about the  $x$ -axis  $= \pi \int_0^1 y^2 dx$ .

The derivation of the equation of the stem curve has been worked in terms of diameters, but in computing the volume of the tree by the above integral  $y$  must be expressed as radius. Since the derived values of  $y$  are percentages of breast-height diameter they will apply as radii just as well as diameters. The limits of integration in our problem are from  $x=0$ , the tip of the tree, to  $x=1$ , breast-height point or unit height, and the result will be in terms of unit radius cubed. The cylinder with which the tree volume is to be compared will have radius 1, altitude 1 and volume of  $\pi$  in terms of unit radius cubed. The absolute form factor will then be—

$$\frac{\pi \int_0^1 y^2 dx}{\frac{\pi}{\int_0^1 \frac{x^2 dx}{(a+bx)^2}}} = \int_0^1 y^2 dx,$$

or since  $y^2 = \frac{x^2}{(a+bx)^2}$ .  $\int_0^1 \frac{x^2 dx}{(a+bx)^2}$  = absolute form factor.

From a table of integrals we find

$$\int \frac{x^2 dx}{(a+bx)^2} = \frac{1}{b^3} \left[ a+bx - 2a \log_e (a+bx) - \frac{a^2}{a+bx} \right]$$

Substituting  $x=1$  gives

$$\frac{1}{b^3} \left[ (a+b) - 2a \log_e (a+b) - \frac{a^2}{a+b} \right]$$

Since we have found that  $a+b=1$  in all cases this expression reduces to

$$\frac{1}{b^3} (1 - a^2) \quad (\text{A})$$

Substituting  $x=0$  gives

$$\frac{1}{b^3} \left( a - 2a \log_e a - \frac{a^2}{a} \right)$$

or

$$-\frac{1}{b^3} (2a \log_e a) \quad (\text{B})$$

Subtracting B from A gives

$$\text{Absolute form factor} = \int_0^1 y^2 dx = \frac{1}{b^3} (1 - a^2 + 2a \log_e a).$$

Absolute form factors derived in this way are included in Table 5, and are plotted in Figure 17 to permit rapid interpolation of the absolute form factor for any desired form quotient. If these absolute form factors are plotted on ratio or semilogarithmic paper they will fall very nearly into a straight line. This means that the form factors and hence the volumes in the portions above breast height are in a geometric progression and the rate of increase per unit increase of form quotient was found from the slope of the line to be approximately

1.9 per cent. The volume differences for the entire stems vary with total height and form quotient, since total volumes are related to the breast-high form factors, and so no general figure can be used with absolute accuracy. However, plotting breast-high form factors on semilogarithmic paper makes it possible to compute the approximate rate of increase of total volume for various ranges of heights and form quotients. It will be found that the rate of increase varies from less than 1 per cent for very short trees in the very low classes up to a little more than 1.6 per cent for the taller trees. A rate of 1.5 per cent represents a good average which will apply quite closely for all trees above 40 feet in total height except those most open-grown individuals. This means that the difference in volume corresponding to a form class interval of five units is about 7.5 per cent.

A method of approximate integration which appears to give slightly better results than that based on the Smalian formula is known as Simpson's rule. By this rule—

$$\int_0^1 y^2 dx = \frac{\Delta x}{3} (y_0^2 + 4y_1^2 + 2y_2^2 + 4y_3^2 + 2y_4^2 + \dots + y_{10}^2)$$

By this rule the 10 sections into which the stem is divided are calculated in pairs according to Newton's prismoidal formula.

A direct expression of the effect of the variations of the different series from the tapers given by the formula may be obtained from the absolute form factors. The difference in absolute form factors between any two series may be converted into percentage difference in volume by dividing the difference in form factors by the form factor of the series used as a basis for comparison. Accordingly, the absolute form factors for the various series, excepting those for hemlock, and the corresponding volume error as compared to the formula, have been computed by the use of Simpson's rule for approximate integration and are tabulated in Table 6, columns 1 to 4. In this table the basis of comparison is the same as that used in Figure 16 (see pp. 698-701) except in a few series, notably those for balsam fir, where the falling off in the tops extends below the middle of the stem. What is wanted here as a basis for comparison is the form class into which the material would fall in practice, when corrected for root swell. Whenever the divergence of the tops from the formula extends below the middle of the stem, as indicated in Figures 11 and 12, the desired form class is represented by a line from breast height through the actual value for the mid-point, rather than by the lines in the figures, for these latter, in order to show directly the relative divergence in the tops of the various species, were drawn to fit the lower portions of the stem alone. When the actual form class of the material, with root swell eliminated, is taken for such series, the diameters in the lower portion of the stem are greater than those given by the formula and hence the volume errors may become positive in sign, even though relatively the series show a greater falling off in the tops than other series with negative errors.

TABLE 6.—Absolute form factors and volume errors

## WESTERN YELLOW PINE

Normal form class (1)	Absolute form factor		Volume error (4)	Basis number of trees (5)	Probable error normal variation (6)	Error above probable error (7)
	Formula figure 17 (2)	Material (3)				
			<i>Per cent</i>			
46 0	0 309	0.313	+1.3	3	1.6	-----
55.6	.371	.369	-.5	6	1.2	-----
60 2	.404	.405	+ .2	17	.7	-----
66.1	.450	.448	-.4	29	.5	-----
70.2	.485	.486	+ .2	55	.4	-----
75.0	.528	.527	-.2	70	.3	-----
79.1	.571	.560	-1.9	18	.7	-1.2
82.6	.610	.594	-2.6	3	1.6	-1.0

## NORWAY SPRUCE (JONSON—1910)

62.2	0.419	0.420	+0.2	10	0.9	-----
70.7	.490	.491	+ .2	30	.5	-----
77.6	.555	.587	+ .4	7	1.1	-----

## SCOTCH PINE (JONSON—1911)

65.6	0.447	0.447	-----	10	0.9	-----
71.9	.500	.498	-0.4	40	.4	-----
75.9	.538	.536	-.4	35	.5	-----
81.1	.592	.592	-----	9	.9	-----

## EUROPEAN LARCH. HEIGHTS 8-14 M. (MATTSSON—1917)

61.8	0.417	0.418	+0.2	50	0.4	-----
63.9	.433	.430	-.7	37	.5	-0.2
66.8	.456	.454	-.4	52	.4	-----
67.9	.465	.464	-.2	54	.4	-----
70.8	.490	.490	-----	49	.4	-----
73.4	.514	.511	-0.6	52	.4	- .2

## EUROPEAN LARCH. HEIGHTS 15-22 M. (MATTSSON—1917)

64.1	0.435	0.437	+0.5	36	0.5	-----
66.7	.455	.454	-.2	42	.4	-----
70.5	.487	.487	-----	30	.5	-----
70.5	.487	.488	+ .2	33	.5	-----
71.9	.500	.502	+ .4	35	.5	-----
75.6	.535	.534	-.2	33	.5	-----

## SIBERIAN LARCH (MATTSSON—1917)

55.4	0.369	0.369	-----	84	0.3	-----
61.1	.411	.410	-0.2	99	.3	-----
65.4	.445	.442	-.7	46	.4	-0.3

## WESTERN WHITE PINE (BEHRE 1923. U. S. F. S. 1910-1913)

55.2	0.368	0.374	+1.6	4	1.4	+0.2
61.4	.413	.412	-.2	25	.6	-----
65.4	.445	.443	-.4	47	.4	-----
69.7	.481	.477	-.8	43	.4	-----
73.7	.516	.515	-.2	16	.7	-----
80.2	.582	.576	-1.0	4	1.4	-----

TABLE 6.—*Absolute form factors and volume errors—Continued*  
 WESTERN WHITE PINE (CLAUGHTON-WALLIN AND McVICKER—1920)

Normal form class (1)	Absolute form factor		Volume error (4)	Basis number of trees (5)	Probable error normal variation (6)	Error above probable error (7)
	Formula figure 17 (2)	Material (3)				
71.2	0.491	0.560	<i>Per cent</i> +1.2	16	6.7	+0.5

## DOUGLAS FIR (U. S. F. S. 1910-1913)

60.4	0.405	0.406	+0.2	5	1.3	-----
67.3	.490	.461	+2	11	.9	-----
70.5	.487	.484	-.6	29	.5	-0.1
75.9	.538	.538	-----	17	.7	-----
80.6	.557	.570	-2.9	10	.9	-2.0

## DOUGLAS FIR (CLAUGHTON-WALLIN AND McVICKERS—1920)

77.4	0.553	0.556	+0.5	11	0.9	-----
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## NORWAY PINE (CLAUGHTON-WALLIN AND McVICKER—1920)

65.8	0.448	0.444	-0.9	11	0.9	-----
70.8	.490	.485	-1.0	30	.5	-0.5
75.0	.529	.522	-1.3	40	.4	-.9

## NORWAY PINE (REED—1924)

70.8	0.499	0.499	-----	21	0.6	-----
77.0	.549	.544	-0.9	15	.7	-0.2

## YOUNG NORTHERN WHITE PINE (CLAUGHTON-WALLIN AND McVICKER—1920)

59.7	0.400	0.399	-0.2	5	1.3	-----
65.7	.447	.443	-.9	15	.7	-0.2
70.8	.490	.481	-1.8	9	.9	-.9

## OLD NORTHERN WHITE PINE (WRIGHT—1923)

71.0	0.492	0.496	+0.8	342	<div style="border-left: 1px dashed black; border-right: 1px dashed black; height: 100px; width: 100%;"></div>	
70.6	.488	.489	+2			
71.5	.486	.491	-1.0			
72.5	.505	.502	-.6			
74.2	.521	.520	-.2			
75.4	.533	.527	-1.1			
76.4	.543	.536	-1.3			
78.8	.547	.538	-1.6			
80.2	.552	.564	-3.1			

## WHITE, RED, AND BLACK SPRUCE (WRIGHT, 1923)

61.1	0.411	0.412	+0.2	865	<div style="border-left: 1px dashed black; border-right: 1px dashed black; height: 100px; width: 100%;"></div>	
62.1	.418	.419	+2			
65.4	.445	.447	+4			
67.1	.459	.459	-----			
70.1	.484	.480	-.8			
72.3	.503	.496	-1.4			
75.4	.532	.528	-.8			
76.8	.547	.535	-2.2			

TABLE 6.—*Absolute form factors and volume errors*—Continued  
WHITE SPRUCE (CLAUGHTON-WALLIN AND McVICKER, 1920)

Normal form class (1)	Absolute form factor		Volume error (4)	Basis number of trees (5)	Probable error normal variation (6)	Error above probable error (7)
	Formula figure 17 (2)	Material (3)				
65.9	0.449	0.450	<i>Per cent</i> +0.2	6	1.2	-----

## VIRGIN RED SPRUCE—MAINE (U. S. F. S. 1902)

52.4	0.349	0.359	+2.9	7	1.1	+1.8
62.0	.418	.417	-.2	29	.5	-----
69.8	.482	.477	-1.0	65	.4	-.6
77.6	.555	.541	-2.5	31	.5	-2.0

## SECOND GROWTH RED SPRUCE—NEW HAMPSHIRE (U. S. F. S. 1903)

72.6	0.506	0.499	-1.4	20	0.6	-0.8
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## RED SPRUCE CULLED FOREST—NEW HAMPSHIRE (U. S. F. S. 1903)

60.8	0.409	0.416	+1.7	6	1.2	+0.5
71.4	.495	.484	-2.2	30	.5	-1.7
78.5	.564	.541	-4.1	8	1.0	-3.1

## RED SPRUCE OLD FIELDS—NEW HAMPSHIRE (U. S. F. S.—1903 AND MEYER, 1924)

55.3	0.368	0.385	+4.6	3	1.6	+3.0
60.3	.405	.406	+2	4	1.4	-----
65.2	.444	.446	+5	22	.6	-----
69.9	.483	.479	-8	39	.5	-3
74.9	.527	.514	-2.5	17	.7	-1.8
78.9	.507	.542	-4.4	7	1.1	-3.3

## BALSAM FIR (WRIGHT—1923)

60.0	0.403	0.407	+1.0	362	-----	
65.0	.441	.439	-.5		-----	
70.6	.488	.478	-2.0		-----	
75.5	.534	.522	-2.2		-----	

## BALSAM FIR, SECOND GROWTH—MAINE (MEYER, 1923)

66.2	0.451	0.452	+0.2	18	0.7	-----
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## BALSAM FIR, CULLED FOREST—MAINE (BEHRE—1924)

66.5	0.454	0.458	+0.9	20	0.6	+0.3
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## BALSAM FIR, OLD GROWTH—MAINE (BEHRE—1924)

55.5	0.370	0.385	+4.1	11	0.9	+3.1
60.3	.405	.413	+2.0	21	.6	+1.4
64.6	.438	.447	+2.1	18	.7	+1.3
69.4	.478	.482	+8	18	.7	+1.1
75.9	.538	.524	-2.6	5	1.3	-1.3

TABLE 6.—Absolute form factors and volume errors—Continued

LONGLEAF PINE (BROWN-1924)						
Normal form class (1)	Absolute form factor		Volume error (4)	Basis number of trees (5)	Probable error normal variation (6)	Error above probable error (7)
	Formula figure 17 (2)	Material (3)				
66.9	0.457	0.455	<i>Per cent</i> -0.4	68	0.3	-0.1
72.6	.506	.505	-.2	439	.1	-.1
77.9	.558	.550	-1.4	156	.2	-1.2
SHORTLEAF PINE (BROWN-1924)						
63.5	0.430	0.428	-0.5	21	0.6	-----
72.4	.504	.503	-.2	217	.2	-----
79.2	.572	.559	-2.3	178	.2	-2.1
LOBLOLLY PINE (BROWN-1924)						
62.7	0.423	0.425	+0.5	48	0.4	+0.2
72.0	.501	.496	-1.0	243	.2	-.8
79.2	.572	.553	-3.3	47	.4	-2.9
GRAY BIRCH (HAWLEY-1923)						
46.5	0.312	0.314	+0.6	5	1.3	-----
50.6	.338	.340	+.6	17	.7	-----
56.0	.373	.373	-----	46	.4	-----
61.9	.417	.413	-1.0	56	.4	-0.6
66.0	.449	.446	-.7	37	.5	-.2
70.8	.490	.485	-1.0	16	.7	-.3

The figures in column 4, Table 6, indicate the net volume errors of each particular series as compared to the formula. An examination of these figures shows a fairly consistent tendency for the errors to increase from the lower to the higher form classes. This is a measure of the effect of the divergence from the straight lines noted in Figures 2 to 14. But these figures afford no basis for an estimation of how much of these errors are really the result of divergence from the formula and how much is simply the result of the normal variations of trees included in a given form class average which will be influenced by the number of trees in the average. Furthermore, they give no measure of the variation of the diameters at any point on the stem as compared to the variations of the mid-points which are arbitrarily limited to the form-class interval.

To determine the importance of the error from these sources an analysis was made of the western yellow and western white pine material, since these species agree very closely with the formula. The diameter at each tenth of the stem of each tree was compared to the diameter given by the formula for a form quotient corresponding to the actual average of the class to which the tree belonged. A given deviation at the base of a tree will have a much greater influence upon the volume than a similar deviation in the upper portions of the stem, and a given deviation in a tree of low form quotient will have a relatively greater weight than in a tree of higher

form quotient because the basis of comparison is relatively smaller. To make the observed deviations comparable they should be expressed in terms of their effect upon volume independent of their position in the tree, or the size or form of the tree. Since the diameters have been expressed on a percentile basis, the effect of size of the trees has already been taken care of. To express the effect upon volume of a given percentile deviation in diameter, independent of its position on the stem, a general expression for the resultant rate of change in volume per unit length can be used. The volume of the tree may be considered as made up of a number of cylinders of varying diameters corresponding to the diameters at different points on the stem. As the altitude of these cylinders approaches zero the sum of their volumes approaches the true volume of the tree. An increase or decrease of diameter at any point on the stem may therefore be considered as affecting a cylinder having a diameter equal to that of the tree at the given point and an altitude equal to the interval between successive measurements, in this case one-tenth of the total height.

Let  $V$  = volume

$Y$  = diameter at any point on the stem

$y$  = deviation of diameter from formula values

$H$  = height or altitude

$n$  = number of trees

$f$  = absolute form factor

$AD$  = average deviation

$\sigma$  = standard deviation

Then, for a cylinder

$$V = \frac{\pi}{4} Y^2 H, \text{ and by differential calculus}$$

$$\frac{dV}{dY} = \frac{\pi}{2} HY = \text{rate of change of volume with change of diameter}$$

$$\text{Since } H = \frac{1}{10},$$

$$\frac{\pi}{20} Y = \frac{\text{change in volume per tenth of length resulting from deviation, } y, \text{ in diameter.}}{\text{deviation, } y, \text{ in diameter.}}$$

$$\frac{\pi}{20} AD_y \quad Y = \text{variability of changes in volume per tenth of length corresponding to deviations in diameter} = \text{average error in terms of volume per tenth of length in estimate of diameter at any point by the equation of the stem curve.}$$

$$V = \frac{\pi}{4} Y^2 H f = \text{volume of tree.}$$

Since total height = 1, D. B. H. = 1, and since the standard deviation is approximately 1.253 times the average deviation,

$$\begin{aligned} \sigma \text{ per } \frac{1}{10} \text{ length in percentage of volume} &= \frac{\frac{\pi}{20} AD_y Y}{\frac{\pi f}{4}} \cdot 1.253 \cdot 100 \\ &= \frac{25.06}{f} AD_y Y. \end{aligned}$$

This last expression gives the standard error in percentage of volume resulting from deviations in diameter at any one tenth of the stem.

In order to compare the variations in diameters at different points on the stem with variations at the mid-point, which are limited by the form-class interval, the standard deviations in volume at each tenth of the stem in the different form classes are shown in Table 7 and Figure 18 for the western yellow pine and western white pine material, these species being selected because they fit the formula well.

TABLE 7.—*Relative effect of variability of upper diameters upon volume estimates by formula, shown in percentage of volume*

WESTERN YELLOW PINE

Form class	Percentage of length from tip to breast height									Num- ber of trees
	90	80	70	60	50	40	30	20	10	
	Standard errors in percentage of volume									
45.....	0.743	1.029	1.032	1.456	0.164	0.965	0.345	0.185	0.077	3
55.....	.464	1.849	.582	.829	.308	.343	.571	.249	.098	6
60.....	1.004	1.041	.986	.800	.511	.642	.658	.245	.157	17
65.....	.763	.803	.690	1.118	.390	.664	.688	.539	.215	29
70.....	.831	.958	.974	.794	.402	.859	.740	.652	.326	55
75.....	.652	.816	.980	.779	.416	.636	.710	.714	.417	70
80.....	.440	.687	.851	.649	.345	.716	1.129	1.220	1.598	18
85.....	.308	.549	.265	.714	.240	.740	2.064	2.486	.954	3
Average and total.....	.723	.890	.927	.832	.407	.714	.757	.685	.466	* 198

WESTERN WHITE PINE

60.....	0.756	0.682	0.880	0.818	0.419	0.567	0.575	0.367	0.207	23
65.....	.731	.889	.794	.794	.428	.726	.749	.580	.307	47
70.....	.646	.771	.771	.562	.422	.729	.750	.734	.299	43
75.....	.392	.690	.608	.498	.313	.554	.651	.802	.486	16
Average and total.....	.661	.790	.776	.678	.410	.680	.711	.634	.315	129

\* Three trees in form class 85 are omitted from the average because this class does not fit the formula in the tops.

From these figures it appears that the variations of the diameters at points between the mid-point and the base or the tip involve volume errors seldom over twice the errors arising from the spread of the form-class interval itself. Furthermore, in series which fit the formula well, deviations in diameters in the lower half of the stem are more important in their effect on volume than those in the upper portion, although the latter are high in absolute values. The extreme similarity of the results for the two species also indicates that variation in this respect is probably identical for all practical purposes in all species, and that an evaluation of the total volume error in estimating upper diameters from the formula may be applied quite generally.

If the deviations at the various points on the stem are uncorrelated, the total error resulting from simultaneous deviations at all points will be given by the formula for the standard deviation of a sum

$$\sigma_s = \sqrt{\sigma_1^2 + \sigma_2^2 + \sigma_3^2 + \cdots}$$

It is obvious, however, that deviations at different sections are often correlated with each other. Trees in conformity with the formula but falling near either the upper or lower limits of the form-class interval will have very high positive correlation between deviations at any pair of sections. On the other hand many trees with positive deviations in the lower portions of their stem tend to have negative errors in their tops and vice versa. In such cases, the correlation between deviations in adjacent section in either upper or lower portions of the stem will be positive, but between a deviation from the upper portion and one from the lower portion the correlation will be negative. In

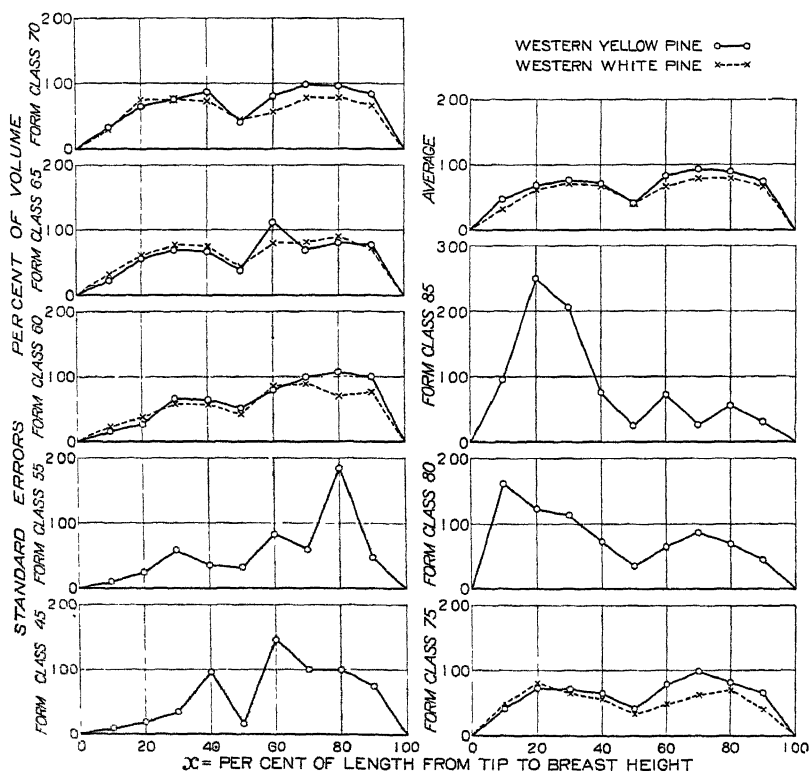


FIG. 18.—Relative effect of variability of upper diameters upon volume estimates by formula

addition there will be many trees which exhibit no correlation at all between deviations at different sections.

To make the formula for the standard deviation of a sum as given above applicable in the case of correlated deviation requires the addition under the radical of as many terms of the form  $2r_{12}\sigma_1\sigma_2$  (in which  $r_{12}$  is the coefficient of correlation between deviations at any two sections) as there are pairs of sections. Since there will be 36 such terms to cover all the possible combinations of 9 sections taken in pairs, this involves a volume of work out of all proportion to the value of the result sought. We are justified therefore in using the simple formula for uncorrelated deviations as the basis for comparison, with an approximate correction for correlation of deviations

applied later to the final average figures. The total standard error in percentage of volume resulting from estimation of all upper diameters from the formula will therefore be approximately:

$$\sigma_s = \sqrt{\Sigma \sigma^2} = \sqrt{\Sigma \left[ \frac{25.06 \, AD_y \cdot Y}{f} \right]^2}$$

To estimate the magnitude of the correction which it is necessary to apply to the results from the approximate formula, to account for correlation of deviations between different sections, average values may be assigned to the factors of the 36 terms of the form  $2r_{12} \sigma_1 \sigma_2$ .

The average product of the standard volume error caused by the individual deviations at the different sections ( $\sigma_1 \sigma_2$ ) may be taken as the square of the average of the errors at the different sections, or  $\left(\frac{\Sigma \sigma}{9}\right)^2$ .

For an approximation of the average value of  $r$  we may assume that any pair of deviations with like sign indicates positive correlation, that any pair of deviations with opposite signs indicates negative correlation, and that the tendency toward negative correlation would offset the tendency toward positive correlation.

This is in effect exactly parallel to the method of expressing correlation by the approximate method of concurrent deviations given by King (16) as—

$$r = \pm \sqrt{\pm \frac{2c - u}{u}}$$

In this formula  $r$  = the coefficient of correlation,  $c$  = number of pairs of deviations with like sign, and  $u$  = total number of pairs in the sample. The  $\pm$  signs both before and under the radical are used to indicate that when the quantity  $\frac{2c - u}{u}$  is negative the coefficient is negative, and when it is positive the coefficient is positive.

To determine the number of pairs of deviations with opposite signs in a given sample, tally each tree according to the number of deviations of positive sign which it exhibits in its nine sections. If  $n_+$  and  $n_-$  represent the number of deviations with positive and negative signs, respectively, in any tree, the number of pairs with unlike sign which may be formed will be given by the formula  $C_{+,-} = n_+ n_-$ , since  $n_+$  positive deviation may be selected and each selection matched with  $n_-$  negative deviation. The total number of pairs of deviations which may be taken from nine sections is given by the formula  $C_{9,2} = \frac{9!}{2! (9-2)!}$ . This has already been given as

36. From the tally of trees according to number of deviations of positive sign and the corresponding numbers of pairs with opposite signs which may be formed, the total number of pairs with opposite sign is obtained. By subtracting this number from the total number of pairs in the sample the number of pairs with like signs is obtained. Substitution in King's formula will then give the value of  $r$ , the net proportion of full correlation. The procedure is illustrated by random samples of 100 western yellow pines and 106 western white pines in Table 8. The net proportion of full positive correlation

exhibited by the western yellow pine is 0.415 and by the western white pine 0.425. It is thus evident that an average value for  $r$  would be somewhere between these figures and that 0.42 would doubtless be a sufficiently conservative figure.

TABLE 8.—*Approximation of average correlation between deviations in diameter at different sections*

Number of sections with + deviation	Number of pairs with opposite sign	Western yellow pine <sup>a</sup>		Western white pine <sup>b</sup>	
		Number of trees	Number of pairs with opposite sign	Number of trees	Number of pairs with opposite sign
9	0	5	0	11	0
8	8	8	64	8	64
7	14	8	112	8	112
6	18	18	324	18	324
5	20	14	280	15	300
4	20	13	260	17	340
3	18	13	234	14	252
2	14	12	168	11	154
1	8	6	48	2	16
0	0	3	0	2	0
-----		100	1,490	106	1,562

<sup>a</sup> Total number of pairs ( $u$ ) =  $100 \times 36 = 3,600$ . Number of pairs with like sign ( $c$ ) =  $3,600 - 1,490 = 2,110$ .  $r = \pm \sqrt{\frac{\pm 2c - u}{u}} = +0.415$ .

<sup>b</sup> Total number of pairs ( $u$ ) =  $106 \times 36 = 3,816$ . Number of pairs with like sign ( $c$ ) =  $3,816 - 1,562 = 2,254$ .  $r = +0.425$ .

From these figures the correction of the formula for the standard deviation of the sum may be approximated as follows:

$$36(2r_{12}\sigma_1\sigma_2) = 36 \cdot 2 \cdot 0.42 \left( \frac{\Sigma\sigma}{9} \right)^2 = .373(\Sigma\sigma)^2$$

The standard volume error for the entire stem resulting from deviations of upper diameters from the equation of the stem curve, corrected for correlation between deviations at different sections, may then be stated as

$$\sigma_s = \sqrt{\Sigma\sigma^2 + .373(\Sigma\sigma)^2} \text{ or}$$

since  $\sigma$  has already been shown to be  $25.06 \frac{AD_v \cdot Y}{f}$

$$\sigma_s = \sqrt{\Sigma \left( \frac{25.06 AD_v \cdot Y}{f} \right)^2 + .373 \left( \frac{25.06}{f} \Sigma (AD_v \cdot Y) \right)^2}$$

The standard errors for entire trees computed separately for each form class by the formula  $\sigma_s = \sqrt{\Sigma\sigma^2 + .373(\Sigma\sigma)^2}$  from the data of Table 7 are shown in Table 9 and Figure 19 for western yellow pine and western white pine. The standard error appears to be almost constant except in the highest form classes of the western yellow pine, where the divergence of the tops from the formula increases the error considerably above what would be expected from the errors

within series conforming more exactly to the formula. In making the calculation for western white pine the data for form classes 55 and 80, four trees in each class, were omitted in order to restrict the calculation to series agreeing very closely with the formula as shown by Table 6. For the same reason the three trees of form class 85 in western yellow pine were omitted from the calculation of the average standard error.

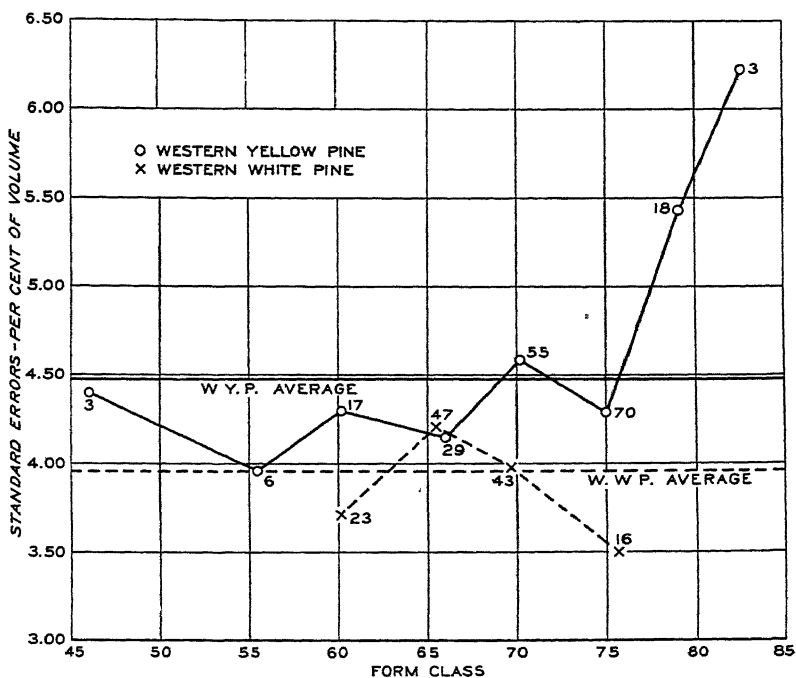


FIG. 19.—Standard errors in volume caused by deviations of upper diameters from the formula

TABLE 9.—Approximate standard errors in percentage of volume caused by deviations of upper diameters from the formula

Form class	Western yellow pine			Form class	Western white pine		
	Actual average form quotient	Standard error volume	Number of trees		Actual average form quotient	Standard error volume	Number of trees
		<i>Per cent</i>				<i>Per cent</i>	
45.....	46.0	4.40	3	60.....	60.1	3.72	23
55.....	55.6	3.96	6	65.....	65.4	4.21	47
60.....	60.2	4.30	17	70.....	69.7	3.98	43
65.....	66.1	4.15	29	75.....	73.7	3.50	16
70.....	70.2	4.59	55				
75.....	75.0	4.29	70				
80.....	79.1	5.43	18				
85.....	82.6	6.22	3				
Average.....	70.44	4.48	198	Average.....	66.92	3.96	129

For the entire 198 western yellow pines with average form quotient of 70.44, the standard error caused by deviations in upper diameters was 4.48 per cent and for 129 western white pines averaging 66.92 in form quotient the standard error from this source was 3.96 per cent. The difference between the figures obtained for the two species may be due in part to the fact that western white pine is in general a smoother, more regularly shaped tree than western yellow pine, but the difference is so slight that it will be safe to assume an average figure for general application.

Taking an average of these figures, say 4.2 per cent, as the standard volume error due to the normal variation of upper diameters of the trees included in any form class, the net volume errors of the different series given in column 4, Table 6, can be modified to determine whether or not the errors indicate significant divergence from the formula. The probable error of an average is given by the formula

$PE_a = \frac{.6745\sigma}{\sqrt{n}}$  in which  $\sigma$  is the standard deviation of the individual trees, 4.2 per cent this case, and  $n$  is the number of trees in the average. Column 6, Table 6, calculated from this formula, gives the probable error in each series attributable to the normal variation of upper diameters. Any volume error exceeding this probable error may be considered as indicative of significant divergence from the formula, since the chances are more than equal that such an error would arise from normal variation of the upper diameters rather than from the sampling alone. On this basis column 7, Table 6, shows the net volume error attributable to divergence from the formula in the different series. It will be noted that for about half the series listed the errors of column 4 are of a magnitude which might result from simple sampling alone, and that errors attributable to divergence from the formula seldom exceed  $\pm 1$  per cent except in the large form classes.

#### ANALYSIS OF VARIATIONS FROM THE FORMULA

In the material presented above a definite and constant tendency was noted in many of the species for the tops in the larger form classes to fall below the values given by the formula. With eastern hemlock the form of the entire tree differs markedly from that given by the formula, but the divergence appears to be an exaggerated case of the type noted for the other species. An attempt has been made to analyze the nature of these variations in a search for a more perfect expression of stem form.

A generalized taper series should give values representing an average for all the species to which it applies, and hence should give lines through the centers of the bands of points in Figure 16, which indicate the spread of values for the various species at each tenth of the stem.

The extremely limited range of the belts of points in the chart and the similarity of form factors as shown above leave little doubt but that all the species studied except hemlock may be measured with a single set of taper curves. The lines representing the formula  $y = \frac{x}{a + bx}$ , however, fall near the upper limits of the belts of points in the top sections of the larger form classes rather than through the centers, and the question therefore arises as to the possibility of

finding an equation more nearly representing the average in all cases without sacrificing the simplicity of the formula already deduced.

By drawing a smooth curve through the center of each belt of points, an average taper series may be obtained. This series may be harmonized by replotting, and used directly as the basis for volume and taper tables, but, inasmuch as the original placing of the curves and their subsequent harmonization would vary with the individual doing the work as well as with the material used as a basis, such a series could never have the nicety and precision of a series based on a mathematical formula nor lend itself so well to analysis and comparison.

Considerable study was, therefore, devoted to the problem of fitting an equation to the average taper series obtained from the chart, but none was found which seemed superior to  $y = \frac{x}{a + bx}$  from the standpoint of simplicity and exactness as well as closeness of fit.

From this study it is quite apparent that any improvement over the equation  $y = \frac{x}{a + bx}$  must involve more than two constants. Inasmuch as the definition of form quotient fixes only two points on the curve, the evaluation of constants in an equation with more than two constants necessitates resort to a method of approximation, and the resulting constants will vary according to the empirical values used in the calculation. Furthermore, in problems such as this, where separate constants must be derived for each form class, the constants should progress systematically from one form class to another. To accomplish this requires plotting the constants derived from the data in order to smooth out the irregularities between form classes. The constants derived by this process are, therefore, subject to variation, and as a result the equation loses much of its value as an exact and precise measure of the stem curve. It is no longer entirely independent of the errors of sampling in the field data nor of personal judgment in manipulation. It does not seem that the slight improvement in closeness of fit and reduction of volume errors in the larger form classes which may be obtained by an equation with more than two constants is sufficient to warrant the sacrifice of the simplicity and exactness of the equation  $y = \frac{x}{a + bx}$ .

It is of interest, however, in support of the general theory that all trees taper in accordance with the same law and that their taper may therefore be expressed by a single type equation, to study the fit of more complex equations to the average series and their adaptability to the peculiarities of the individual species. In this investigation a modification of the original equation, such as  $x/y = a + bx + c \log x$  or  $x/y = a + bx + ce^{dx}$ , seems to give the most satisfactory fit under all conditions.

Between these two equations there seems to be little choice from the standpoint of ability to fit closely any of the individual series. For some species the former type is slightly superior and for others the latter more truly represents the shape of the curves. In attempting to evaluate the constants to fit the average series derived graphically it was found exceedingly difficult if not impossible to harmonize the constants from one form class to the next with the use of the

equation  $x/y = a + bx + ce^{dx}$ , but little difficulty was encountered in deriving a satisfactory set of constants for the equation  $x/y = a + bx + c \log x$ . For either type equation, however, constants may apparently be found which will give values exceedingly close to any of the individual taper series examined, including hemlock. This seems to indicate that the minor variations noted between different species are all of the same general type and that all may be expressed in terms of the same mechanical law.

Using the constants given in Table 10, which were derived from the graphic average series as outlined above, taper series have been computed for the equation  $x/y = a + bx + c \log x$  and the results are shown in Figure 16 for comparison with the original equation and the material.

TABLE 10.—*Constants for  $x/y = a + bx + c \log x$* 

Form class	a	b	c	Form class	a	b	c
45.....	1.222	-0.222	0.000	70.....	0.515	0.608	-0.0615
50.....	1.000	.000	.000	75.....	.448	.716	-.0820
55.....	.822	.186	-.0030	80.....	.393	.812	-.1025
60.....	.696	.346	-.0210	85.....	.347	.898	-.1225
65.....	.596	.486	-.0410				

To show how slight is the effect on volume computations of the refinements gained by this series, the absolute form factors have been computed by Simpson's rule for approximate integration and are given in Table 11 together with those derived from the equation  $y = \frac{x}{a + bx}$ . The constant  $c$  has no appreciable value until form class 60 is reached, so that up to this point there are no differences in the absolute form factors.

TABLE 11.—*Comparison of absolute form factors*

Form class	Absolute form factors		Difference	Volume difference
	$x/y = a + bx + c \log x$	$y = \frac{x}{a + bx}$		
60.....	0.4032	0.4031	+0.0001	<i>Per cent</i> +0.02
65.....	.4413	.4414	-.0001	-.02
70.....	.4816	.4827	-.0011	-.23
75.....	.5257	.5261	-.0024	-.46
80.....	.5742	.5792	-.0050	-.86
85.....	.6276	.6385	-.0109	-1.71

It will be noted that up to form class 75, which is seldom if ever exceeded as an average in forest stands, there is not over 0.5 per cent difference in the total cubic volumes as measured by the two equations and, as the tapers vary most widely in the tips, a large part of this small volume difference lies in the unmerchantable portions of the stem.

#### EFFECT OF VARIOUS FACTORS UPON THE FORM CURVE

In the outline of the development of the form-class system in Sweden it was pointed out that previous to 1911 European foresters had established the fact that taper based on form quotient was

independent of diameter, height, locality, and, to a certain extent, species. On the other hand Wright (26), after considerable work with Canadian species, states that taper within a given form class varies with species, diameter, age, and probably height also. Baker (1) and Behre (4) have both shown that the differences found by Wright practically disappeared when the distortion of the breast-high-diameter by root swell was eliminated from the material. The wide variety in the character of the material included in the present study tends to confirm the European conclusions in this respect, yet it is worth while to inquire more in detail into the effect of various factors upon the form curve in order to check up on this point.

The western yellow pine and red spruce data are especially suitable for this analysis because they cover such a wide range of conditions. With the aid of mechanical sorting and tabulating machines, these

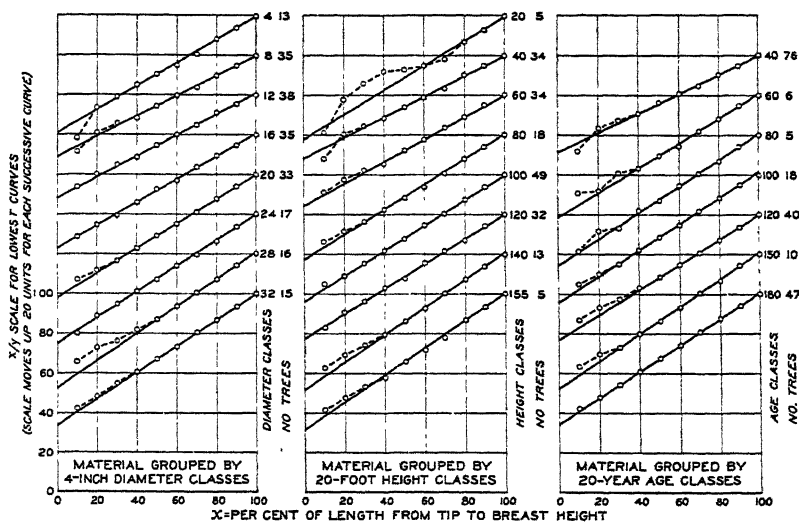


FIG. 20—Effect of diameter, height, and age on form curve of western yellow pine

data were therefore sorted and averaged separately for each of the factors for which notes or measurements were available.

The method of analysis used for each factor is illustrated by the following instance of grouping by diameter classes. All the trees of a given species were first sorted into 4-inch d. b. h. classes. The percentile tapers were then added together for each 4-inch class to get an average taper series for each class regardless of form quotient. For the series thus obtained corresponding values of  $x/y$  were computed, and these were then plotted to note their conformity to the formula and for comparison with one another. If the form curve of the species varies with diameter the smaller trees will exhibit in this plotting a different tendency from that of the larger trees. Comparison of the tendency exhibited by these series with those evident in grouping by other factors gives further indication of the effect of the various factors upon the form curve.

Figure 20 shows the result of averaging the western yellow pine material by diameter, height, and age. No important or consistent

divergence from the straight lines are discernible in any of the groupings, so that we may conclude that in general the conformity of western yellow pine to the formula is independent of diameter, height, and age.

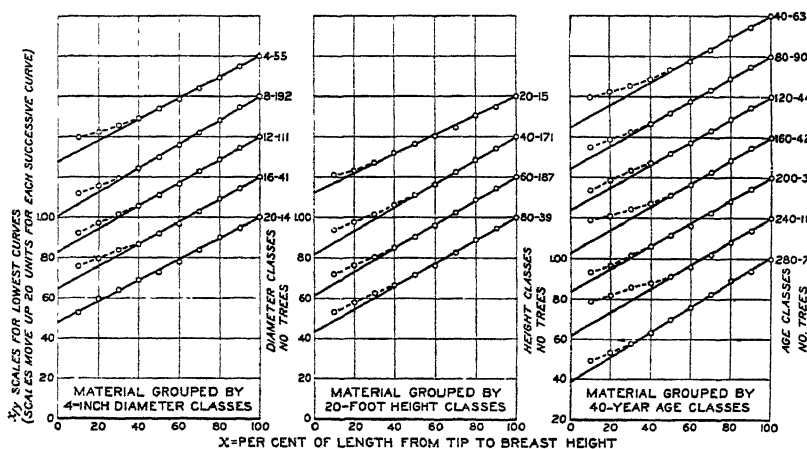


Fig. 21.—Effect of diameter, height, and age on form curve of red spruce

Figures 21 and 22 show the result of averaging the red spruce data by diameter, height, age, crown class, forest type, and site. In practically all the series this species shows a divergence of the tips below the formula but the differences in the amount and character of this divergence are very slight in most instances.

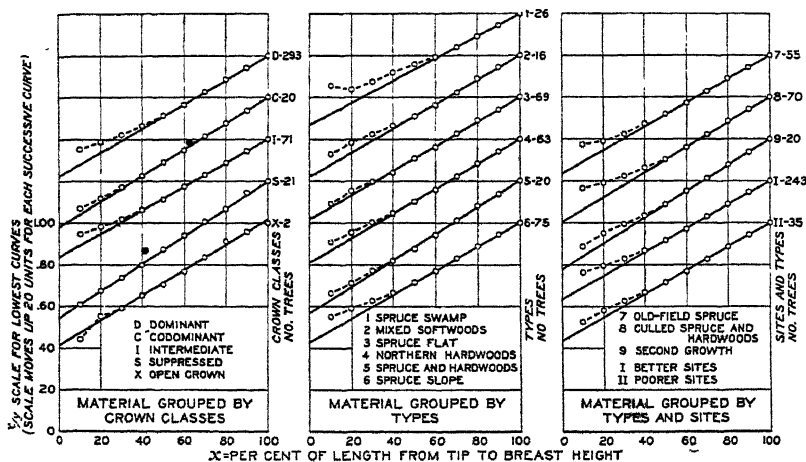


Fig. 22.—Effect of crown class, forest type, and site on form curve of red spruce

From the grouping by diameter classes it appears that the larger sizes hold their taper somewhat better in the tips than the small and average size classes.

The type of form curve appears in no way affected by height or age.

In the grouping by crown classes the suppressed trees hold their taper in the tips much better than the other classes and the greatest divergence of the tops from the formula occurs in the dominant class. The fact that the open-grown trees do not show any divergence of the tops from the formula as might be expected in comparison with the dominant class is hardly significant in view of the small number of trees upon which the figures are based.

In the grouping by forest types the terminology is in accordance with the definitions of forest types adopted by the New England Section of the Society of American Foresters (9) except for Nos. 8 and 9. Type 8, "culled spruce and hardwoods," was differentiated from type 5, "spruce and hardwoods," in order to bring out any difference in form which may result from the changed conditions under which the residual stand develops after a heavy culling. Type 9, "second growth," was differentiated to cover a number of trees so designated on the original tally sheets, which probably represent second-growth stands resulting from fire, windfall, or other natural phenomena, differing radically in character from the usual old growth of the various types but still not partaking of the characteristics of the old-field type.

The divergence of the tips below the formula is most pronounced in the culled spruce and hardwoods type, and considerably more than the average in old-field and spruce-swamp types. But none of these variations are of much importance, nor are they in excess of similar divergencies appearing at random in some of the other groupings. It may be noted, however, that figures from western white pine left after selective logging 15 years previously showed a very similar tendency to differ slightly in form from trees grown in undisturbed forest conditions throughout their existence. This is doubtless the result of an entirely different distribution on the stem of the wood produced each year, caused by the sudden change in exposure following opening up of the stand. It is also reasonable to suppose that as the trees develop further they will again adjust themselves to the new conditions in accordance with the mechanical laws which control the distribution of wood, and eventually regain a form curve similar to that found in natural and undisturbed stands.

On better sites the divergence of the tops below the formula appears greater than on poorer sites, but again the difference is not important.

From this summary we may conclude that, although minor tendencies to differ in form type may be observed, none of them are of sufficient importance to warrant special treatment; and accordingly for all practical purposes we may consider tapers based on the formula to apply equally well under all conditions. It should be noted, however, that these analyses apply only to the type of form curve and that nothing is indicated here as to the effect of the various factors upon the average form quotient or upon the thickness of bark and amount of root swell at the breast-height point. These matters are the subject of further investigations now under way.

#### DEVELOPMENT OF TOTAL CUBIC FOOT VOLUME TABLES

Total cubic foot volume tables may be derived from the absolute form factors in Table 5 by the method used by Jonson (13) and outlined in Chapman's *Forest Mensuration* (6), which involves the

derivation of breast-high form factors as the basis for computation of cubic contents.

In this procedure the normal diameter at the ground is calculated by prolonging the equation of the stem curve down to the ground separately for each height class. In the same way a new middle

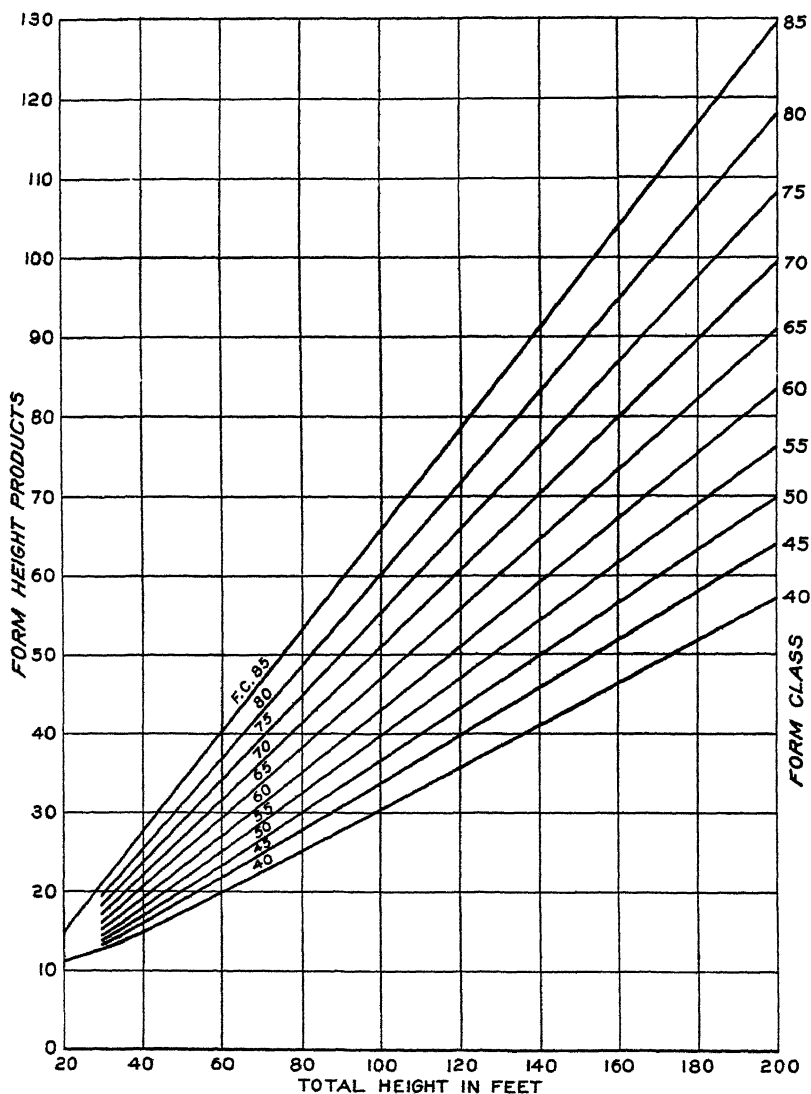


FIG. 23.—Form-height products based on  $y = \frac{x}{a+bx}$  for total cubic contents inside bark

diameter is computed for each height class on the basis of total height. These values give a new set of absolute form quotients for which corresponding absolute form factors are interpolated from the curve of Figure 17. These absolute form factors are then converted into breast-high form factors.

From a set of breast-high form factors computed in this way form-height products were derived by multiplying each factor by its corresponding height. These form-height products are shown in Figure 23. They are based on total cubic foot contents of the stem without bark. The chart permits interpolation of the form height products for trees of any height from 20 to 200 feet and for any form class from 40 to 85. In the construction of this chart calculations of the form height products were made only for height classes 20, 25, 30, 40, 60, 80, 120, 160, and 200 in form classes of 5 units each.

The next step is to multiply the form-height products by the basal areas corresponding to the different inch classes for the range likely to be needed in any particular case. The resulting volume tables will show the contents of trees according to their normal diameter inside bark, and will serve for all species which conform approximately to the type equation  $y = \frac{x}{a + bx}$ . This step can be accomplished most economically by plotting the tables on logarithmic coordinate paper. Since the plotting of volume on diameter on logarithmic paper gives a series of straight lines for different height classes, the lines can be accurately placed from the computation for only two or three selected diameter classes.

The final step in the completion of the tables must be taken separately for each species (or group of species) because of differences in bark thickness and amount of root swell at breast height; for practical reasons the final volume tables should be based on the usual breast-height measurement rather than normal diameter inside bark. The normal diameters corresponding to the different d. b. h. classes must, therefore, be determined separately for each species. With this relation worked out, as will be illustrated later, the transformation to the final volume tables can best be done on the logarithmic plotting of the tables for each form class, cubic foot contents on normal diameters with a curve for each height class, by drawing vertical lines on the abscissae corresponding to even inches of d. b. h.

#### TAPER CHARTS FOR DIAMETER, HEIGHT, AND FORM CLASSES

As a basis for volume tables in board feet, ties, poles, or other units of product it is necessary to convert the percentile taper series of Table 5, derived from the generalized equation, into taper tables or charts giving diameters and heights in absolute rather than relative terms. To work out arithmetically from the percentile taper series the diameters at specified heights for all sizes and form classes required in a volume table is quite tedious and serves only the purpose of that particular table. At any time when it may be desired to make a new table using different standards of utilization or based on a different class of products the entire job may have to be repeated.

It is, therefore, not only desirable but economical to put the taper tables into permanent form for general use with all species to which the basic equation of the stem curve applies. This can be done to best advantage by a graphic method suggested by Hohenadl (12).

The percentile tapers from Table 5 are first plotted on logarithmic coordinate paper and smooth curves drawn through the points for

each form class. These curves are then transferred to a sheet of transparent tracing paper. The actual diameters in inches at any height in feet for trees of any size can now be read directly by shifting the curves on the respective axes for diameters and heights. This procedure is illustrated by Figure 24, in which only form class 70 is shown. Curve I shows the preliminary plotting of the percentile tapers which is transferred to tracing paper.

If the percentile tapers are multiplied by the normal diameter at breast height, 16 inches for example, the result will be actual diameters in inches at the respective percentages of the tree's height above breast height. This is accomplished graphically by shifting the curve in the direction of the diameter axis until the breast-height point ( $x=1.00$ ) corresponds to the normal diameter in question, 16 inches (Curve II). In this transformation the logarithm of 16 is added to the logarithm of each of the percentile taper series.

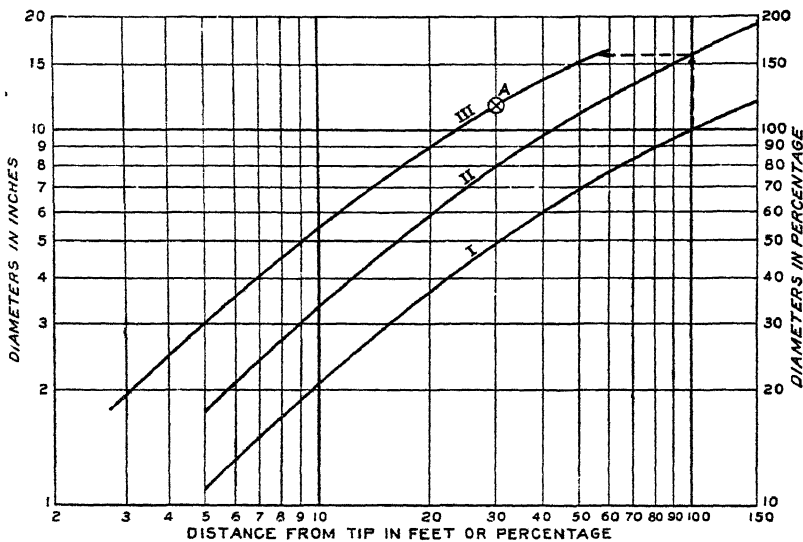


FIG. 24.—Conversion of percentile taper curves to absolute values

To convert the percentile heights at which the diameters are now expressed to actual heights in feet is accomplished in a similar manner by shifting the curve in the direction of the height axis until the breast-height point corresponds to the total height of the tree less 4.5 feet. Thus from Curve III we read the diameter 30 feet from the tip of a 60-foot tree with normal diameter of 16 inches as 11.7 inches. It is necessary to note that the height scale is for distances from tip, so that to read scaling diameters at different heights above ground requires first the simple conversion of the desired heights to distances from tip for each height class.

The conversion of the percentile taper series into tables of taper for different diameter height and form classes may also be accomplished by means of an alinement chart<sup>5</sup> designated to solve the equation of

<sup>5</sup> For a discussion of the theory of alinement charts, see: (5, 8, 17).

the stem curve,  $y = \frac{x}{a+bx}$ . Such a chart has been worked out and is presented as Figure 25. This chart permits direct reading for any value of the form quotient, rather than for certain arbitrary classes, but it is not as convenient to read as the logarithmic chart.

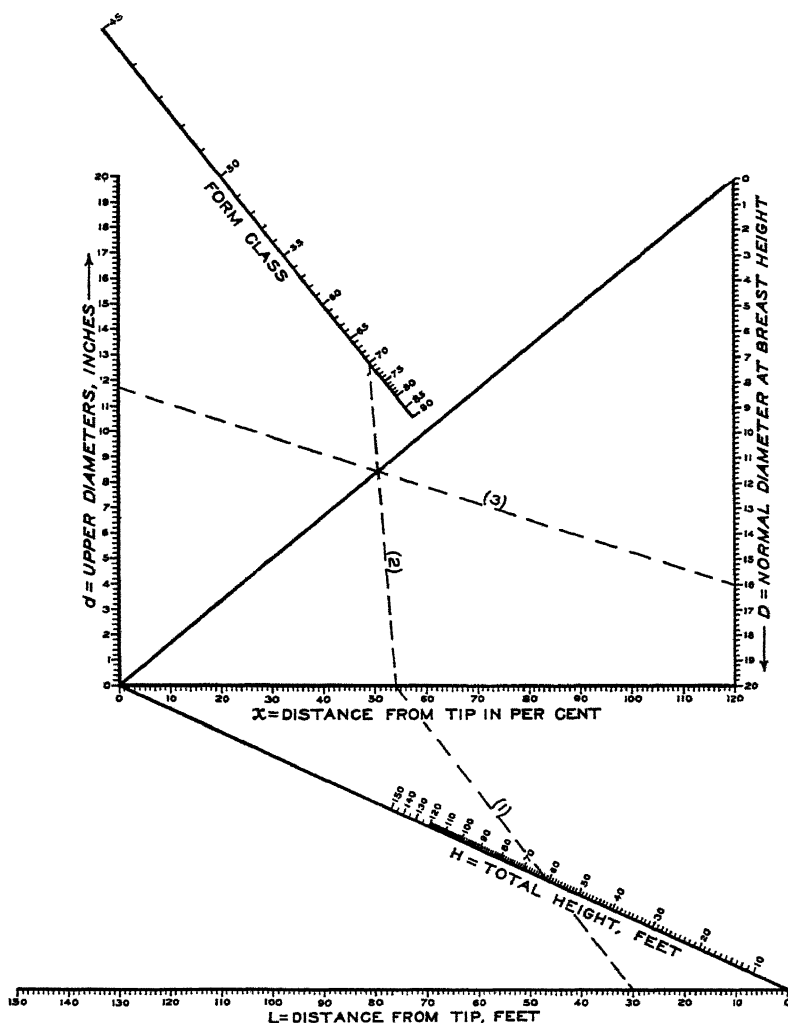


FIG. 25.—Alignment chart for  $\frac{d}{D} = \frac{x}{a+bx}$ , giving the diameter at any point on the stem of trees of any diameter, height, and form class

The complete solution of the equation from the chart is best accomplished with the aid of two silk threads attached to small pins at either end, working with the chart fastened upon a drawing board.

The equation  $\frac{d}{D} = \frac{x}{a+bx}$  may be rewritten in the form  $\frac{d}{D} = \frac{x}{1+b(x-1)}$ ,

since  $a+b=1$  in all cases. In this form the equation has four components and can be solved with a pair of intersecting lines on the alinement chart. An extra operation is required, however, to get values for the component  $x$ .

The first operation gives the value for  $x$  and involves expressing the distance from the tip at which the diameter is to be determined as a percentage of the height above breast height. This is accomplished by stretching one of the strings from the given point on the scale "distance from tip, feet" across the value corresponding to the total height on the diagonal scale for height, to its intersection with the horizontal scale "distance from tip in per cent," at which point a pin should be placed. The broken line (1), Figure 25, shows that 30 feet from the tip of a 60-foot tree is 54 per cent of the distance from tip to breast height.

Now stretch the thread from the pin already placed on the  $x$  scale across the required form class on the diagonal scale calibrated for this variable and pin it in this position. Broken line (2) illustrates this for form class 70 and the value of  $x$  given by line (1).

Now stretch the second string from a given value on the vertical scale for "normal diameter at breast height," across the intersection of the fixed thread and the unlabeled diagonal line, to the vertical scale for "upper diameters," from which the value sought may be read. In the illustration, broken line (3) shows that the upper diameter 30 feet from the tip of a 60-foot tree of form class 70 is 11.7 inches when the normal diameter at breast height is 16 inches. The upper diameters for the given distance from tip, total height, and form class can now be read for trees of any diameter by rotating the thread for line (3) about the intersection of line (2) and the unlabeled diagonal, and reading the upper diameters corresponding to successive normal diameters at breast height. Just as in the case of the cubic foot volume tables, the relation between actual diameter breast high and normal diameter must be worked out before the chart can be used for any specific table.

## II. APPLICATION TO WESTERN YELLOW PINE

The application of the general form-class taper charts and volume tables to the measurement of stands of timber of any given species requires, first, a study of the relation between the normal diameter inside bark at breast height and the actual breast-high diameter for the species in question; second, a study of the variability of the form quotients and absolute form factors of the trees in a stand in order to justify the use of an average form quotient for the entire stand; and, third, a method of determining the average form quotient of the stands to be estimated in the field so that the proper form-class table may be selected for each set of conditions.

These factors, being inherent in the timber itself, enter into the application of any system of volume tables, and a method of analysis which will shed light on any of them will aid materially in improving the technic of timber estimating.

The following sections are not presented with any sense of finality but are merely intended to illustrate the problems to be met and to suggest in the light of work already done—especially that in Sweden, briefly reviewed in the introduction to this paper—what appears to

be the most practical method of handling the application of the general form-class volume tables. Further study is required to give more adequate knowledge of the fundamental principles involved and of the best method of procedure in working out the relations for any specific case, but the results shown for the limited amount of western yellow pine material available are in general agreement with the Swedish work, in so far as they are comparable, and also with fragmentary investigations of other species in this country.

REDUCTION OF BREAST-HIGH DIAMETER FOR DOUBLE BARK THICKNESS AND BUTT SWELL

The simplest method by which the reduction of actual breast-high diameters for bark thickness and butt swell can be taken care of is to determine an average value of the required reduction for each diameter class and apply these figures quite generally over the entire range of the species in question. If it can be shown that the sum of double bark thickness and root swell at breast height is closely enough correlated with the breast-high diameters so that the normal diameters inside bark may be estimated from the latter with a reasonable degree of accuracy, this method will be quite satisfactory for all practical purposes.

Accordingly the sum of double bark thickness and root swell at breast height for each tree was tabulated for the computation of the coefficient of correlation. (Table 12 and fig. 26.) The amount of root swell was in each instance determined as previously described by plotting the original tapers on cross-section paper and extending the normal convex curve of the upper part of the stem down to breast height whenever the root swell extended above this point.

TABLE 12.—Correlation of double bark thickness and butt swell at breast height to breast-high diameter; western yellow pine

Double bark thickness and butt swell— Inches ( <i>y</i> )	Diameter at breast height—Inches ( <i>x</i> )																			Total
	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	44		
0.5-0.9	8	5	1																14	
1.0-1.4	1	2	16	3	3			1											25	
1.5-1.9		1	6	9	3	2	5	3											27	
2.0-2.4				6	6	1	6	2	1	3									26	
2.5-2.9				1	2	4	4	6	3					2	1				19	
3.0-3.4					1	1		6	2	5	1	1							17	
3.5-3.9							2	2	4	2	1	1	1	1	1				16	
4.0-4.4							1	1	2	4	1	2	1	3					15	
4.5-4.9									5	1				1					7	
5.0-5.4							1		1	1				2	1				6	
5.5-5.9														3	1	1			5	
6.0-6.4										1				1			1		3	
6.5-6.9														2	1				4	
7.0-7.4									1				2						3	
7.5-7.9																				
8.0-8.4																1		1	2	
8.5-8.9																				
9.0-9.4																	1	1	2	
Total	9	8	23	19	15	8	19	15	19	17	3	6	11	10	3	3	1	1	190	

$M_x=16.80$  inches;  $M_y=2.96$  inches;  $\sigma_x=8.16$  inches;  $\sigma_y=1.73$  inches;  $r=0.8530 \pm .0198$ ;  $\eta=0.865 \pm .0182$   
 $b=0.186$ ;  $S_y=\pm .9290$ ;  $y=.186x-0.16$ .

From this table the coefficient of correlation was found to be  $+0.853 \pm 0.0198$ . This correlation appears fairly satisfactory and

seems to indicate that the relation between the breast-high diameter and the corresponding reduction for bark thickness and butt swell may be expressed graphically as a straight line.

To further test this point the correlation ratio corrected for number of arrays<sup>6</sup> was also computed. If the difference between the squares of the correlation ratio and the coefficient of correlation is found to be insignificant, the relation may be assumed to be rectilinear. The correlation ratio was found to be  $\pm 0.865 \pm 0.018$ .

The difference between the squares of the correlation ratio and the coefficient of correlation is 0.0206 and the standard error of this difference is  $\pm 0.0208$ . Since the difference is no greater than its standard error it can not be considered significant and a straight line will express the desired relation.

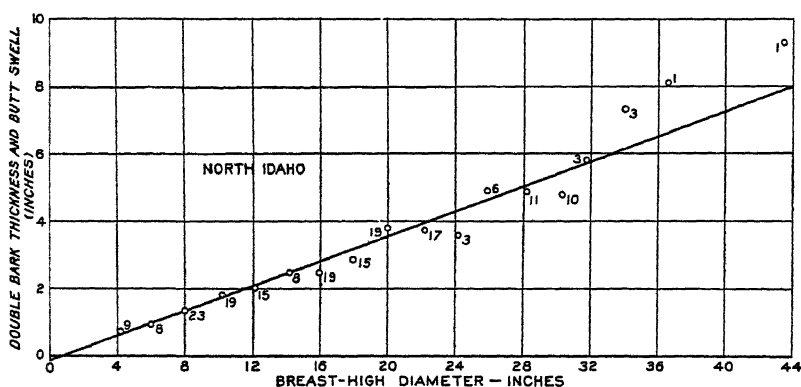


FIG. 26.—Reduction of breast-high diameter for double bark thickness and butt swell, western yellow pine, north Idaho

The formulae and symbols used in these and succeeding calculations are as follows:

$d$  = deviation from approximate mean of variable denoted by subscript

$d_o$  = deviation of approximate mean from true mean

$n$  = number of observations

$\Sigma$  = sign of summation

$m$  = mean of variable denoted by subscript

$\sigma$  = standard deviation of variable denoted by subscript  

$$= \sqrt{\frac{\Sigma d^2}{n} - d_o^2}$$

$r$  = coefficient of correlation =  $\frac{\Sigma d_x d_y - d_{ox} d_{oy}}{n \sigma_x \sigma_y}$

$s_r$  = standard error of coefficient of correlation =  $\frac{1 - r^2}{\sqrt{n}}$

<sup>6</sup> For an explanation of the "correlation ratio" see (22, 27).

$\sigma_{my}$  = standard deviation of means of  $y$  arrays

$$\eta_o = \text{correlation ratio} = \frac{\sigma_{my}}{\sigma_y}$$

$$\eta = \text{correlation ratio corrected for number of arrays} = \sqrt{\frac{\eta_o^2 - \frac{k-3}{n}}{1 - \frac{k-3}{n}}}$$

$k$  = number of arrays

$$s_\eta = \text{standard error of correlation ratio} = \frac{1 - \eta^2}{\sqrt{n}}$$

$$s_{\eta^2 - r^2} = \text{standard error of } \eta^2 - r^2 = 2 \sqrt{\frac{\eta^2 - r^2}{n}}$$

$b$  = regression coefficient of bark thickness and butt swell on breast-high diameter =  $r \frac{\sigma_y}{\sigma_x}$

$s_y$  = standard error in estimating  $y$  from  $x$  by regression equation  
 $= \sigma_y \sqrt{1 - r^2}$

The regression equation for estimating double bark thickness and butt swell from breast-high diameter was computed from the above correlation as  $y = 0.186x - 0.16$ .

The standard error in estimating the double bark thickness and butt swell of individual trees from their breast-high diameter by this relation is  $\pm 0.93$  inches. But for volume table purpose we are not interested in the estimation of the normal diameter for each individual tree, but desire only to know the average normal diameter corresponding to each d. b. h. class. The error for such class averages will be reduced in proportion to the square root of the number of trees in the average, so that for 20 trees the standard error would be about 0.21 inches. Since the variability of the observations increases with increase in diameter, and since the error likely to be incurred would be more serious for small trees than large ones, it is desirable to express this error as a percentage of the normal diameter obtained from the estimate, rather than in inches regardless of size as above.

To do this the reduction of breast-high diameter for each tree was computed from the regression equation. These were then compared with the actually measured reductions and the deviations expressed as percentages of the calculated normal diameters. From these percentile deviations, the standard deviation was found to be  $\pm 5.55$  per cent. This means that the standard error in calculating the normal diameter for any tree from the regression equation  $y = 0.186x - 0.16$  will be  $\pm 5.55$  per cent.

The same result may be approximated without the arithmetic of computing the deviations for the individual trees by simply expressing the standard error of estimate from the regression equation (0.93 inch) as a percentage of the average diameter of the material (16.8 inches).

To express the error in estimation of normal diameter in terms of effect upon volume we may follow a procedure similar to that used for errors due to variation of upper diameters (p. 710).

Thus for the volume of the tree

$$V = \frac{\pi}{4} Y^2 H f$$

$$\frac{dV}{dY} = \frac{\pi}{2} Y H f = \text{rate of change of volume with change in normal diameter.}$$

Since normal diameter,  $Y=1$  and since  $H=1$ ,

$$V = \frac{\pi}{4} f, \text{ and } \frac{dV}{dY} = \frac{\pi}{2} f,$$

$$\frac{\pi}{2} f.d = \text{change in volume for deviation } d.$$

and

$$\frac{\pi}{2} f \sigma_d = \text{standard volume error arising from deviation in normal diameter.}$$

Therefore

$$\frac{\frac{\pi}{2} f \sigma_d}{\frac{\pi}{4} f} = 2 \sigma_d = \text{standard error as percentage of volume due to deviation in normal diameter.}$$

The standard error in percentage of volume corresponding to a standard error  $\pm 5.55$  per cent in the estimation of the normal diameter of individual trees will therefore be  $\pm 11.1$  per cent.

Of course it is not probable that this relation will prove to be rectilinear for all species, but the general procedure illustrated by the western yellow pine material may still be followed. First, determine whether there is a satisfactory correlation and whether the relation is rectilinear by computing the coefficient of correlation and the correlation ratio. Then fit a curve to the diameter class averages by use of the regression coefficient if rectilinear and graphically if curvilinear.

Further study may indicate the desirability of including some other factor than d. b. h. in the estimation of the double bark thickness and root swell, but nothing definite is available on this point as yet.

#### USE OF AVERAGE FORM QUOTIENT FOR ENTIRE STAND

The use of an average form quotient for all the trees in a stand is justified by the assumption that positive errors in measuring trees with form quotients below the average will be offset by negative errors in measuring trees with form quotients above the average. This hypothesis will hold good if the form of the trees in the stand is independent of their size and if the average form quotient used corresponds to the average absolute form factor of the trees in the stand and hence to the tree of average volume.

Material from two entirely different types of western yellow pine stands was used to study these points. The first of these stands was typical open virgin growth, partly pure and partly mixed with white fir (*Abies concolor*) and Douglas fir, on good rolling agricultural soil near Worley, Idaho. The second type of stand was fully stocked even-aged second growth, 35 to 55 years old, along the foothills north

of Moscow, Idaho. Forty-eight tree measurements were taken at random from the virgin stand and 47 from the second growth.

The coefficient of correlation between form quotient and diameter was  $-0.228 \pm 0.138$  in the virgin stand and  $-0.453 \pm 0.117$  in the second growth. These figures indicate practically complete independence between form and diameter in the virgin stand but a slight tendency for the larger trees to have lower form quotients in the even-aged second growth.

The form of the trees within a stand is also practically independent of height, for the coefficient of correlation between form quotient and height was only  $+0.259 \pm 0.136$  for the virgin stand and  $+0.0245 \pm 0.147$  in the second growth.

These results check with the work of Mattsson, who states that the range of form quotients in trees of the average class is as great as that in the entire stand.

It has previously been shown that the absolute form factors are not proportional to the form quotients, but vary in geometric progression with a ratio of increase of approximately 1.9 per cent per unit of form quotient. However, since this ratio of increase is so

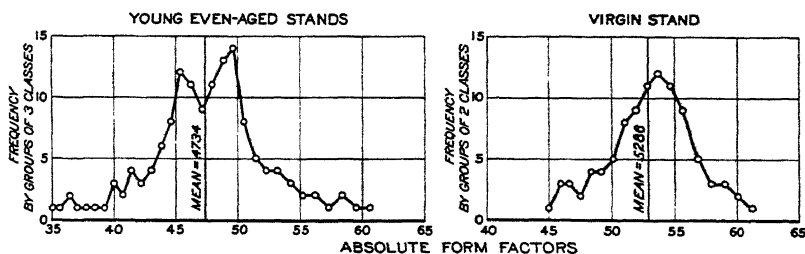


FIG. 27.—Dispersion of form factors in stands of western yellow pine

low, the curve of form factors on form quotients does not depart widely from a straight line within the range of values ordinarily encountered in a stand and so the difference in weight to be given to trees above and below the average form quotient will not be important. Furthermore, the slight error involved from this cause will be offset in some cases because the larger trees tend to average slightly lower in form quotient. Thus the larger trees should be given less weight in an average based on form quotient because of their slightly lower form quotient but more weight because of their greater diameter. For a check on the importance of this point the distributions of absolute form factors and form quotients were analyzed in the two types of stand already described. These distributions are presented in Table 13 and Figure 27. The distributions for both are slightly skew but less for the form factors than for the form quotients. For the virgin stand the actual average form quotient was 74.9 and the form quotient corresponding to the average absolute form factor was 75.1. For the even-aged second growth these figures were 68.6 and 68.8, respectively. The differences are entirely insignificant and the distributions are so nearly normal that we may safely use a direct average of the form quotients and apply the normal laws of error in evaluating the precision of the average.

TABLE 13.—*Dispersion of form quotient and form factors in western yellow pine stands*

## YOUNG EVEN-AGED STANDS

Form quotient	Number of trees	Moving average of three classes			Form quotient	Number of trees	Moving average of three classes		
		Median form quotient	Number of trees	Absolute form factor			Median form quotient	Number of trees	Absolute form factor
52.0-52.9		52.5	1	0.351	68.0-68.9	3	68.5	9	0.471
53.0-53.9	1	53.5	1	.357	69.0-69.9	1	69.5	11	.479
54.0-54.9		54.5	2	.364	70.0-70.9	7	70.5	13	.488
55.0-55.9	1	55.5	1	.371	71.0-71.9	5	71.5	14	.496
56.0-56.9		56.5	1	.378	72.0-72.9	2	72.5	8	.505
57.0-57.9		57.5	1	.385	73.0-73.9	1	73.5	5	.514
58.0-58.9	1	58.5	1	.393	74.0-74.9	2	74.5	4	.523
59.0-59.9		59.5	3	.400	75.0-75.9	1	75.5	4	.532
60.0-60.9	2	60.5	2	.407	76.0-76.9	1	76.5	3	.542
61.0-61.9		61.5	4	.414	77.0-77.9	1	77.5	2	.552
62.0-62.9	2	62.5	3	.422	78.0-78.9		78.5	2	.562
63.0-63.9	1	63.5	4	.430	79.0-79.9	1	79.5	1	.573
64.0-64.9	1	64.5	6	.438	80.0-80.9		80.5	2	.584
65.0-65.9	4	65.5	8	.448	81.0-81.9	1	81.5	1	.595
66.0-66.9	3	66.5	12	.454	82.0-82.9		82.5	1	.606
67.0-67.9	5	67.5	11	.462					

## VIRGIN STAND

Form quotient	Number of trees	Moving average of two classes			Form quotient	Number of trees	Moving average of two classes		
		Median form quotient	Number of trees	Absolute form factor			Median form quotient	Number of trees	Absolute form factor
66.0-66.9	1	66.0	1	0.450	75.0-75.9	6	75.0	11	0.523
67.0-67.9	2	67.0	3	.458	76.0-76.9	6	76.0	12	.537
68.0-68.9	1	68.0	3	.466	77.0-77.9	5	77.0	11	.547
69.0-69.9	1	69.0	2	.475	78.0-78.9	4	78.0	9	.557
70.0-70.9	3	70.0	4	.483	79.0-79.9	1	79.0	5	.568
71.0-71.9	1	71.0	4	.492	80.0-80.9	2	80.0	3	.579
72.0-72.9	4	72.0	5	.501	81.0-81.9	1	81.0	3	.590
73.0-73.9	4	73.0	8	.510	82.0-82.9	1	82.0	1	.601
74.0-74.9	5	74.0	9	.519			83.0	1	.613

Direct average form quotient:

Young even-aged stands..... 68.6

Virgin stand..... 74.9

Form quotient corresponding to average form factor:

Young even-aged stands..... 68.8

Virgin stand..... 75.1

In the virgin stand the form quotients showed a range of 16 units, from 66 to 82, with a standard deviation of  $\pm 3.7$  units. In the second growth the range was 28 units, from 53 to 81, with a standard deviation of  $\pm 5.6$ . This higher variability is doubtless due in large measure to the more heterogeneous character of the stands from which the trees are selected. Eliminating only 3 of the most extreme trees reduces the range to 21 units, from 58 to 79, and brings the standard deviation down to  $\pm 4.5$ . In both stands the dispersion followed closely the normal frequency distribution, and the range of form quotients in trees of average diameter was as great as the range in the entire stand. These figures are in close agreement with similar studies made elsewhere, as shown in Table 14, and a standard deviation of  $\pm 4.5$  in form quotient may be taken as an average for general

use. Since each form-quotient unit corresponds to approximately 1.5 per cent of total volume, the standard volume error in the use of an average form quotient for the entire stand will be  $\pm 6.75$  per cent.

TABLE 14.—Dispersion of form quotients within the stand

Species	Reference	Range				Standard deviation form-quotient units
		Num-ber of trees	Form-quotient units	Mini-mum form quotient	Maxi-mum form quotient	
Western yellow pine:						
Virgin stand.....		48	16	66	82	$\pm 3.7$
Young stand.....		44	21	58	79	$\pm 4.5$
Western white pine.....	Behre (MISS.)	28	22	52	74	$\pm 4.5$
European larch.....	Mattsson (1917)		25			$\pm 4.4$
Jack pine.....	Wright (1923)	206	28	58	86	$\pm 3.9$
Northern white pine.....	do	100	22	59	81	$\pm 4.5$

## ESTIMATION OF AVERAGE FORM QUOTIENT

For the estimation of the average form quotient of stands in the field the indirect method based on the form-point idea which has been developed in Sweden and outlined in the introduction to this paper should be applicable to any conditions. The use of this method requires the working out of a relation between form quotient and form point. For this purpose a procedure similar to that used in working out the reduction of breast-high diameter for double bark thickness and butt swell was employed. Measurements of form point were available for only 138 tress. Of these 48 were taken in different parts of a mature stand of varied density and mixture, 50 were taken on average trees selected from a number of even-aged second-growth stands covering a wide range of ages and sites, and 40 were deduced from measurements of crown length on trees from young second growth and young merchantable timber. The data are tabulated for calculation of correlation in Table 15 and shown graphically in Figure 28.

TABLE 15.—Correlation of form point and form quotient; western yellow pine

Form quotient $\times 100$	Form point—(percentage of total length from tip to nearest 0.5)											Total
	13.0-17.5	18.0-22.5	23.0-27.5	28.0-32.5	33.0-37.5	38.0-42.5	43.0-47.5	48.0-52.5	53.0-57.5	58.0-62.5	63.0-67.5	
45.1-47.5.....											3	3
52.6-55.0.....								1		1		2
55.1-57.5.....									1	1		2
57.6-60.0.....					1	2	3		1			7
60.1-62.5.....					1	1	3			2		8
62.6-65.0.....				1	1			1	1			3
65.1-67.5.....			1	2	1	1	2	2				9
67.6-70.0.....			2	4	3	2	1					12
70.1-72.5.....	2	2	3	3	15	1	1					27
72.6-75.0.....		4	6	13	4	3	1					31
75.1-77.5.....	2	2	7	8	2	3						24
77.6-80.0.....		2	3	2								7
80.1-82.5.....				1	1							1
82.6-85.0.....			1	1								2
Total.....	4	10	23	35	28	13	11	4	3	4	3	138
Actual average form point.....	15.0	20.9	25.3	29.9	35.3	40.2	44.5	49.4	55.3	59.7	65.0	-----
Acting average form quotient.....	74.5	74.9	74.5	73.6	70.4	69.5	64.7	62.4	59.0	57.7	46.6	-----

The coefficient of correlation was found to be  $-.752 \pm .037$  and the correlation ratio, corrected for number of arrays,  $-.788 \pm .032$ . Although the plotting of the averages, as in Figure 28, indicates some tendency toward curvature, the difference between the squares of the correlation ratio and coefficient of correlation is only 1.38 times its standard error, and as this is hardly sufficient to warrant the assumption of a curvilinear relation a straight-line relationship may be used with sufficient accuracy. The regression equation computed on this basis is—

$$y = -.4966x + 87.22, \text{ which for practical use may be rounded off as } y = -.05x + 87.$$

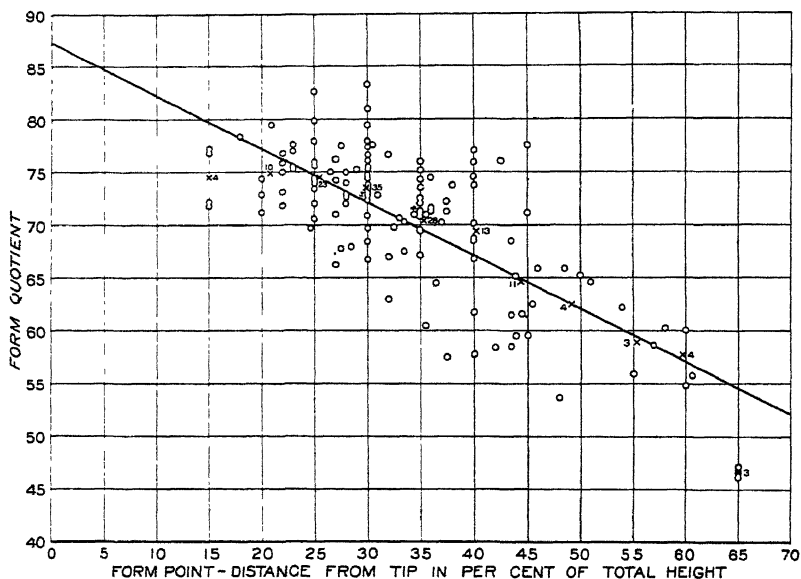


FIG. 28.—Correlation of form point and form quotient; western yellow pine, north Idaho; basis, 138 trees

The standard error of estimating the form quotient of an individual tree from its form-point height by this equation is  $\pm 4.63$ , which would result in a standard error of cubic volume estimate of about 6.94 per cent. Just as in the reduction of breast-high diameter for double bark thickness and butt swell, however, this relation will be used for estimating the average form quotient of a stand from its average form-point height, rather than for individual trees. The standard error of such an estimate may be derived by statistical method. The standard error in estimating the form quotient of individual trees from their form points ( $\pm 4.63$  units) is in fact the standard error of the observations of the individual form quotients, the true variability of which has been shown to be about  $\pm 4.5$  units. The standard error of the observed values will then be—

$$\sigma_{x_1} = \sqrt{\sigma_x^2 + \sigma_d^2}$$

in which  $x_1$  = observed deviation,  $x$  = true deviation and  $d$  = error of

observation. From this the standard deviation of an average from quotient based upon measurement of form point on  $n$  trees is—

$$\sigma_{ax_1} = \frac{\sigma_{x_1}}{\sqrt{n}} = \sqrt{\frac{\sigma_x^2 + \sigma_a^2}{n}}$$

The number of trees which should be measured as the basis for determination of the average form quotient will therefore depend upon the limit or error which is allowed the average. If the standard error of the average is set at  $\pm 1$  form-class unit, which is equivalent to saying that the chances are only 1 to 80 that the observed average form quotient will fall more than half the form-class interval above or below the true average, it will be necessary to measure the form point on more than 40 trees. Thus—

$$\text{If } 1 = \sqrt{\frac{4.5^2 + 4.63^2}{n}}, \\ n = 41.69$$

The estimation of the average form-point height in the field does not involve any difficulty. It is best accomplished with the aid of a Christen hypsometer calibrated uniformly into tenths so that, when the total height of the tree is just included between the upper and lower notches of the instrument, the relative height of any point on the stem can be read directly as a percentage of the total height. The relative height of the form point is measured with this instrument on a number of trees of normal development selected at random in the stand and these measurements are averaged. The experience of the writer, working with a number of assistants in these stands of western yellow pine and also in timber of other types, confirms the conclusion that different persons will agree so closely in their estimate of form point in the stand that personal judgment offers little difficulty. The writer believes that in practice the forest will not have to be subdivided any further for the application of the form-class principle than is customary in the segregation of types and age classes. In other words, it is thought that a separate estimate of form quotient would only be required for such changes of type or age class as are ordinarily recognized in timber cruising.

The writer also believes that further study of variations in average form under different conditions will make it possible to use, without preliminary estimation, a single form-class table for timber of a given species in certain broad, easily recognized categories throughout its range. His observations in this respect agree with Wright (26) who states that all mature stands of northern white pine will fall into form class 70 and that slow-growing black spruce as a type would fall into form class 68. Mature stands of western yellow pine in north Idaho, it is believed, will fall quite consistently into form class 75.

It may be of interest to note that the relation established by statistical method for western yellow pine checks almost exactly with the writer's preconceived notion gained in measuring plots for a yield study in even-aged timber. In this work the average form class for each stand was estimated by eye, and from this the average form point determined. On many occasions a few sample trees were cut and measured in detail. It soon appeared that form class 75 corresponded to a form point 25 per cent from the tip of the tree and that each

increase of 5 per cent in the distance of the form point from the tip corresponded to a decrease of 2.5 form-class units. The statistical result could not have been biased by this notion because most of the trees from which the correlation was computed were measured before the idea had been formulated and in entirely different stands, the form points being measured entirely independently and without any idea of what the form quotients would prove to be.

It is of further interest to compare these results with the form-point relation developed by Jonson (15) in Sweden. Jonson's figures fall into a slight curve with form quotients from 3 to 5 units below the western yellow pine regression line. As pointed out in the introduction to this paper Mattsson (20) found Jonson's function about 2 units low for European larch, Petrini (23) found it equally low for Norway spruce and (24) about 6.5 units low for Lapland pine. From the absolute form factors it has been shown that each unit of form class corresponds to a cubic volume difference of about 1.5 per cent. These results suggest that, although it will be necessary to study the form-point relation for each species separately, it may eventually be found that a single function will hold good for many species.

#### NET ERROR OF ESTIMATE. VOLUME TABLE CHECKS

The method of application of the universal form-class tables having been explained and figures indicative of the errors to be expected in each step in the case of western yellow pine given, it remains to evaluate the net error from all sources which may be expected in the use of this system and to check this theoretical measure of accuracy by tests on representative stands.

Where a species, such as western yellow pine, has a form curve in conformity with the formula errors in estimating volume by the form-class system based on this formula arise from four sources.

In the first place, an error may be expected in the estimate of any tree or stand from the use of an average form quotient for the entire stand. Then an error will arise from the fact that this average form quotient can not be determined by direct measurement, but is estimated indirectly from the average form-point height. The third source of error is the use of an average reduction of the breast-high diameter in each size class for bark thickness and butt swell. Finally, an error is to be expected from the normal variation of upper diameters within the arbitrary form-class interval of five units.

From the foregoing discussion the errors in the estimate of individual trees in any western yellow pine stand are summarized in Table 16.

TABLE 16.—Standard errors in estimation of volume of individual trees by universal form-class volume table system; western yellow pine

Source of error	Standard error	
	Unit measured	Equivalent volume
Use of average form quotient for entire stand.....	±4.5 F. Q. units.....	<i>Per cent</i> ±6.75
Estimate of average form quotient from average form point of 42 trees.....	±1.00 F. Q. units.....	±1.50
Estimate of normal diameter from d. b. h.....	±5.55 per cent normal diameter.....	±11.10
Normal variation of upper diameters within the form-class interval.....	±4.2 per cent volume.....	±4.20
Net error from all sources.....		±13.74

The net error from all sources is calculated according to the formula—

$$\sigma_s = \sqrt{\sigma_1^2 + \sigma_2^2 + \sigma_3^2 + \sigma_4^2}$$

in which  $\sigma_1, \sigma_2, \sigma_3, \sigma_4$ , are the standard errors from each source separately. This gives a net standard error of approximately 13.74 per cent.

The error in estimating the volume of an entire stand may be derived from these figures by reducing each error proportionately to the total number of trees in the stand—except that the error in the estimation of average form quotient from form point will be the same in the estimate of the entire stand as for any individual tree in the stand unless a greater number of observations are made. On this basis the net standard errors in the estimation of entire stands are given in Table 17 for stand samples of three sizes.

TABLE 17.—Standard errors in estimation of volume of entire stands by universal form-class volume table system; western yellow pine

Source of error	Percentage of volume by number of trees in stand		
	100	200	1000
	<i>P. ct.</i>	<i>P. ct.</i>	<i>P. ct.</i>
Use of average form quotient for entire stand.....	0.68	0.48	0.21
Estimate of average form quotient from average form point of 42 trees.....	1.5	1.5	1.5
Estimate of normal diameter from d. b. h.....	1.11	.78	.35
Normal variation of upper diameters within the form-class interval.....	.42	.30	.13
Net error from all sources.....	2.03	1.78	1.56

From the table it appears that ordinarily the most important source of error in applying this system arises from the estimation of the average form quotient of the stand. Using 40 or more trees for this purpose results in a net standard error in the estimate of stands containing more than 100 trees of less than 2 per cent. Increasing the number of trees upon which the form point is measured to 100, as would be entirely feasible in a strip estimate of a large area, reduces the error to a little over 1 per cent.

To check the above theoretical figures by actual tests on typical samples, the two sets of western yellow pine material already described on page 730 may be used.

The normal diameter for each tree was computed from its actual breast-high diameter with a slide rule. The form height for each tree was read from Figure 23 for the even form class corresponding most closely to the average for the stand. These form heights were then multiplied by the basal areas corresponding to the normal diameters. The total volume was corrected 1.5 per cent for each unit of form quotient by which the actual average of the stand differed from the even form class used in the computation. This result was compared to the actual volume of the trees computed by the Smalian formula for the sections above breast height and with the portion below breast height considered as a cylinder. The deviations of the individual tree volumes corrected in the same way for average form quotient were also computed and averaged.

For the 47 trees from even-aged second-growth stands the actual total volume was 363.66 cubic feet. Their volume was estimated from the formula as described above as 355.87 cubic feet. The difference amounts to  $-2.14$  per cent. The average deviations of the individual trees was 8.81 per cent with the positive and negative errors well distributed over the range of diameter classes.

Since in this check the error in estimating the average form quotient of the stand was eliminated by using the actual average form quotient of the material, the theoretical error to be expected would be derived from the other figures of Table 16 by reducing them in proportion to the square root of the number of trees. The standard error of the aggregate estimate of 47 trees is  $\pm 1.95$  per cent. On the same basis the theoretical standard error in the estimate of individual trees becomes 13.6 per cent, which is equivalent to an average deviation of about 10.8 per cent. Thus, although the actual aggregate error is a trifle greater than the theoretical standard error, the actual average deviation is less than the theoretical average deviation and the individual errors are well distributed as to sign, so that the check may be considered satisfactory.

The actual volume of 49 trees in the virgin stand was 7,654.35 cubic feet and the estimate from the formula gave 7,722.05, or an aggregate difference of  $+0.88$  per cent. The average deviation of the individual trees was 12.4 per cent. For 49 trees the theoretical standard error is  $\pm 1.95$  per cent. In this case the actual aggregate error is well within the theoretical standard error, and although the average deviation is slightly greater than the theoretical average error, since the positive and negative errors are well distributed over the range of diameter classes, the check is on the whole satisfactory.

The virgin stand from which these trees were selected was differentiated into two fairly distinct types. About half of the trees came from a shallow draw in which the yellow pine was mixed with other species such as white and Douglas firs. The other half came from adjacent stands in which the timber was pure yellow pine of typical parklike, open character. The trees from each of these types were checked separately to see what difference might be revealed.

In the pure stand 24 trees showed an aggregate error of  $-7.42$  per cent, which is 2.7 times the theoretical standard error of  $\pm 2.78$  per cent. The average deviation was 11 per cent and the majority of the deviations were negative.

In the mixed type 25 trees showed an aggregate error of  $+6.77$  per cent, or 2.5 times the theoretical standard error of  $\pm 2.73$  per cent. The average deviation was 14.5 per cent and nearly all the errors were in the positive direction. From these figures it is evident that the mixed stand of virgin western yellow pine differs consistently in some manner from the pure type.

Further examination of the original data indicates that the reason for the large errors in estimating these virgin stands lies in the estimate of normal diameters from the actual breast-high diameters. It appears that in the mixed type the butt swell averaged consistently above the average while in the pure type it ran slightly below the average. In view of this it may be necessary to have separate curves of normal diameter on breast-high diameter for different types or sites; but the available western yellow pine data are not adequate to settle this point.

It is important to note that the failure of the form-class tables, as used in this example, to measure satisfactorily the individual components of the data from the virgin stand of western yellow pine does not constitute a valid argument against the system as a whole. In fact, it serves to illustrate one of the main advantages of the system. It is obvious from the figures given that no single volume table could measure both the pure and the mixed stand accurately, since the differences are inherent in the timber itself and not in the tables used for its estimation. By the form-class percentile taper system these differences can be analyzed and the errors ascribed to their respective causes, whether they be due to differences in the form curve of the trees as a whole, variations in form quotient, or variations in thickness of bark and amount of butt swell at breast height. The author already has under way in spruce and balsam fir in the northeast more detailed studies of the factors influencing bark thickness and butt swell and the variations of form under different conditions.

### SUMMARY

#### I. GENERALIZED TAPER CURVES AND VOLUME TABLES

Unsatisfactory results in the application of volume tables in this country may be due primarily to failure of the tables to recognize differences in form quotient and variations in bark thickness and butt swell at breast height.

A highly satisfactory system of timber estimating has been developed and used in Sweden upon the basis of a formula derived by Höjer to express the general law of stem taper.

In the present studies diameters are measured at intervals of 10 per cent of the height of the tree above breast height. Distortion of butt swell is eliminated graphically from the breast-height measurement, and all diameters are expressed as a percentage of the normal diameter at breast height. Grouping may therefore be done independent of size upon the basis of normal form quotient, which is the ratio of the diameter inside bark half way between breast height and the tip and the normal diameter at breast height.

Analysis of western yellow pine material showed that Höjer's equation gave values for the upper sections of the larger form classes far above those actually attained by the trees. This tendency had also been noted by other investigators, notably in the cases of Scotch pine and European larch in Sweden, and northern white and Norway pines in Canada.

By methods of graphic analysis a new equation, having the form  $y = \frac{x}{a + bx}$ , was fitted to the western yellow pine material. In this equation  $x$  = distance from the tip, expressed as percentage of total height above breast height, at which diameter,  $y$ , expressed as percentage of normal diameter at breast height, is measured. A series of tapers fitting this equation will fall into a straight line when values of  $x/y$  are plotted on  $x$ .

The equation  $y = \frac{x}{a + bx}$  was found to fit the following species fairly closely: Norway spruce, Scotch pine, European larch, Siberian larch, western white pine, Douglas fir, Norway pine, northern white pine,

white, red, and black spruce, balsam fir, longleaf pine, shortleaf pine, loblolly pine, and gray birch. For several of these species the values given by the formula are still somewhat higher than those attained by trees of the larger form classes in the upper portions of their stems, but the new formula comes very much closer to approximating the general average form than that devised by Höjer and has the further advantage of being very much easier to work with. Eastern hemlock was the only species differing widely from the formula.

Cubic volume increases geometrically with form quotient with a change of 1.9 per cent of volume above breast height and an average of 1.5 per cent of total volume corresponding to each unit of form quotient. The standard error in percentage of volumes due to the normal variation of upper diameters of the trees included within a form class interval of five units is approximately  $\pm 4.2$  per cent.

Comparison of the absolute form factors for the different species with those corresponding to the formula, after making allowance for the standard error to be expected from normal variation of upper diameter within the form class interval (Table 6), shows that for most of the species analyzed the divergence of the tops of the trees from the values given by the formula does not give rise to significant volume errors and that significant errors of more than  $\pm 1$  per cent are seldom found except in the larger form classes.

An attempt to modify the basic equation to give a better average fit in the tops of trees with large form quotient gave unsatisfactory results, because no equation could be found which combined a better fit with the simplicity and ease of handling of the equation  $y = \frac{x}{a + bx}$ .

Comparison of absolute form factors with those of an equation of the type  $x/y = a + bx + c \log x$ , which gives a very close approximation of the average tapers of all the species analyzed, shows that up to form class 75, which is seldom if ever exceeded as an average in forest stands, there is not over 0.5 per cent difference in the total cubic contents as measured by the two equations.

Calculating the tapers of western yellow pine separately by diameter, height, and age, and of red spruce separately by diameter, height, age, crown class, forest type, and site showed no significant differences in the form curve from any of these factors. The most noticeable variation appeared in the red spruce from heavily culled stands and from old-field growth, where the tops fell somewhat further below the values given by the formula than is characteristic for this species under other conditions.

Tables of total cubic-foot volume are constructed from the percentile taper series by the method used by Jonson and outlined in Chapman's "Forest Mensuration". This leads to a set of volume tables based on normal diameter, height, and form class applicable to any species conforming to the formula. Final tables for any individual species are derived from these general tables by reading the normal diameter corresponding to each actual breast-high diameter class for the species in question.

The percentile taper series are easily converted into absolute values for different normal diameters and heights with the aid of logarithmic coordinate paper, or an alignment chart may be used to calculate graphically the diameters at any point on the stem for trees of any diameter, height, and form class.

## II. APPLICATION TO WESTERN YELLOW PINE

The application of volume tables derived from the formula by this method requires a calculation of the average reduction of breast-high diameter for double bark thickness and butt swell for different species and an estimation of the average form quotient of each stand.

The average reduction of breast-high diameter for double bark thickness and butt-swell was derived from the coefficient of correlation and may be expressed as  $0.186 \text{ d. b. h.} - 0.16$ . The standard error resulting from estimating the normal diameter of an individual tree from its actual breast-high diameter is  $\pm 5.55$  per cent of the desired normal diameter, which is equivalent to  $\pm 11.1$  per cent of volume. It is possible that different relations will have to be developed for differences in site or type of timber.

The form quotients of the trees composing any stand are practically independent of diameter, height, and species. The standard deviation of the form quotients within a stand is generally about  $\pm 4.5$  units for any species.

Although form quotients are not exactly proportional to absolute form factors, they are so nearly so that an average form quotient may be used safely for the entire stand. The standard volume error in estimating the volume of individual trees from the average form quotient of the stand is  $\pm 6.75$ .

The form-point system may be used for estimating the average form quotient of the stand. The standard error in estimating the form quotient of individual trees from their form-point heights is  $\pm 4.63$  units. Since each unit of form quotient is equivalent to about 1.5 per cent of volume this would result in a standard error of  $\pm 6.94$  per cent in cubic-volume estimate.

Measurements of form point on 42 trees in the stand are necessary to attain a standard error in the average form quotient of not over  $\pm 1$  form-class unit.

Experience may enable cruisers to judge average form class of stands of timber from the physiognomy of the forest. A separate estimate of the form class should only be necessary for such changes of type or age class as are ordinarily recognized in timber cruising.

The net standard error from all sources in estimating the volumes of individual western yellow pine trees by the universal form-class volume table system is  $\pm 13.74$  per cent. In applying this system to entire stands with samples of over 200 trees and average form quotient determined by form-point measurement of at least 40 trees, the standard error is about  $\pm 2$  per cent.

## CONCLUSIONS

The fundamental principle upon which this system is based, namely, that taper expressed in relative rather than absolute terms is independent of diameter and height for trees of a given form class, was established by European research 17 years ago and has been repeatedly checked since that time.

The further extension of this principle which postulates that taper within a given form class is also independent of species is shown to hold true within certain limits when butt swell is eliminated. Individual species show characteristic differences in taper principally

in the upper portions of their stems, but for most commercial conifers these differences are not sufficient to justify independent treatment.

This permits the use of tables calculated from a mathematical formula and based upon the normal diameter at breast height for a large number of species and eliminates a large part of the labor in volume table construction, at the same time supplying much stronger tables than can be produced by the old system.

The application of these general tables involves a separate study of the reduction of the breast-high diameter for bark thickness and butt swell for each species. This factor is chiefly dependent upon the breast-high diameter itself, but further study is required to discover what other factors may have a measureable influence upon it.

The underlying principle of generalized percentile taper series may be applied with almost equal economy and precision whether or not use is made of a formula for the stem curve. Neither is it necessary to differentiate form classes in the final tables for field application. Tables based on average form for stands of certain characters or for different sites may be derived just as with the old conventional system but without working up an entirely new set of material for each new table. Baker (2) has outlined a method of using the percentile curve idea without reference to a formula for the stem curve and without differentiating form quotients in the final tables.

The following are the chief advantages of the generalized percentile taper curve system of volume table construction. Of the points listed, the first four apply especially to the preparation or testing of basic tables; it will require further study to establish completely their validity with respect to the determination of normal diameters at breast height.

1. A relatively small number of trees is required as a basis for dependable tables, because the data are not subdivided into size classes before averaging and therefore fewer trees are needed to get strong averages.
2. The tables are just as strong for extreme values in either diameter or height as they are for size classes well represented in the data.
3. The tables can be extended beyond the range of the data with almost as much certainty as attaches to the main body of the table.
4. The technic of preparing the tables is extremely simple and remarkably free from errors of individual judgment in the placing of curves, etc., numerous graphical checks insuring the accuracy of the arithmetical work.
5. Subordinate tables for different units of product or different standards of utilization may be prepared with a minimum amount of labor.
6. The system provides a basis for analyzing the disturbing factors in the use of volume tables and permits the evaluation of errors from various sources separately. A definite check for similarity of form is provided for testing the application to

any stand of timber and the usual volume checks are, therefore, susceptible of more accurate interpretation. No difficulties in the use of this system have yet been suggested which are not inherent in the timber itself and so equally a source of uncertainty in any system of volume tables. This system gives each factor due consideration, thus bringing the disturbing elements to light and paving the way for improvement of timber estimating technic. Little help can be expected from other systems which ignore these disturbing elements altogether.

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# THE EFFECT OF ABSORPTION BY PLANTS ON THE CONCENTRATION OF THE SOIL SOLUTION<sup>1</sup>

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## INTRODUCTION

In many irrigated regions the force of circumstances operates in the direction of requiring the most economical and efficient use of irrigation water. In some situations the area of available land is greater than the supply of water. In others, where water must be lifted to reach the land, the cost of pumping is a powerful incentive to sparing use. There are still other situations where it is believed that the extravagant application of water contributes to the saturation of the subsoil, a condition popularly known as water-logging. Many of the troubles that occur on irrigated land are believed to be due either directly or indirectly to the excessive use of irrigation water.

Although there are many cogent reasons for advocating the most sparing use of water in irrigation, there are also reasons why enough water should be used to prevent the accumulation of injurious quantities of soluble salts in the root zone of the soil. Nearly all irrigation waters carry in solution appreciable quantities of salts. Certain of these salts are absorbed by crop plants to a limited extent, but others are used very little if at all. Where the system of irrigation is such that all the water applied to the soil is held within the root zone, the salts brought to the land by the irrigation water remain in the root zone, largely dissolved in the solution.

There are two assumptions with respect to irrigation practice that are very generally accepted but which do not appear to be well supported. These may be stated as follows:

(1) That crop plants absorb the soil solution, including both the water and its dissolved salts, substantially as it occurs in the soil.

(2) That the ideal system of irrigation is one in which only enough water is applied to the soil to moisten the root zone to its water-holding capacity, in order to supply the needs of crop plants and to meet the unavoidable losses of direct evaporation.

These two assumptions are very closely related. The second is really based on the first. If the first is not well founded, the second is certainly open to question.

Persons who have studied the physiology of plants or who are familiar with the literature of that subject do not generally hold the view that plants absorb the soil solution as it exists in the soil. To them the phenomenon of selective absorption is readily accepted as a fact. They conceive that a plant may absorb water or any dissolved electrolytes or gases according to its needs and almost if not quite independently of the relative abundance of the various constituents in the solution. Numerous experiments have been made, the results of which support this view. For the most part these

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experiments have been designed to show the selective absorption by plants as between certain electrolytes in the solution, and it seems generally to have been taken for granted that the rate of water absorption is also independent of the rate of absorption of the dissolved substances. From what is known of the phenomena of absorption by plants it is entirely conceivable that a crop plant might absorb water from a soil solution and at the same time not absorb corresponding quantities of the substances dissolved in that solution. In other words, a plant having its roots in contact with a soil solution might, during its period of growth, modify the character of the solution in the direction of increased concentration—a result similar to that which would follow if water were lost from the solution by evaporation.

Although such a result of absorption by plants is entirely in accord with known facts, there does not appear to be in the literature of the subject very much in the way of direct evidence on this point. For that reason it seemed desirable to conduct experiments that should yield such evidence. The experiments described in the following pages were designed with that end in view. They were planned in detail by the writer but were conducted by James F. Breazeale at the University of Arizona, who prepared and analyzed the culture solutions and who was also responsible for the care of the cultures and for making the various observations, except the conductance determinations, which were made by H. V. Smith, also of the University of Arizona.

#### PURPOSE OF THE EXPERIMENTS

The experiments were planned to give unequivocal answers to the following questions:

1. Does the plant absorb its nutrient solution as the solution is presented to the roots?
2. If water and electrolytes are absorbed from the nutrient solution at different relative rates, are these rates influenced by the concentration of the nutrient solution?

These questions may be stated in another way:

3. Does the plant, when its roots are in contact with a concentrated nutrient solution, absorb the water and the electrolytes at the same rate?
4. If, even from a dilute solution, the water is absorbed faster than the electrolytes, is the difference in rate of absorption greater as the solution is made more concentrated?

For the information of those who may not have the time or the inclination to read the details of the description of the experiments and the discussion of its results, it may be said here that questions 1 and 3 are answered in the negative and questions 2 and 4 in the affirmative.

#### DESCRIPTION OF THE EXPERIMENTS

The experiments involved the use of 25 seedlings of barley or wheat, grown as one lot, with their roots immersed in 950 c. c. of nutrient solution. During the period of the experiment the seedlings were supported on a perforated disk covering the glass jar containing the solution. This disk was covered with a soft wax in such a way as to hold the seedlings in position and at the same time to seal the

mouth of the jar and prevent the loss of water from the solution by direct evaporation.

The seedlings were obtained by sprouting a large number of seeds on perforated aluminum disks floating in tap water. When the seeds were well sprouted, with plumules about an inch long, it was possible to pick out lots of 25 that were apparently uniform in size and vigor. These were then transferred to the nutrient solutions and sealed in. The cultures were grown during the latter part of March, 1927, in a greenhouse at the university at Tucson, Ariz.

The culture solutions were obtained in the following manner: A quantity of surface soil taken from a field near Jaynes Station in the Santa Cruz Valley, near Tucson, Ariz., was leached with distilled water through a filter. The percolate was diluted to have a concentration of total salts estimated as approximately 0.5 per cent. This percolate was then analyzed, with the result shown in the second column of Table 2. It was also tested electrically for its specific conductance at 25° C. and for its alkalinity as expressed by the  $P_H$  scale. One portion was tested in its natural condition and another portion after it had been boiled and its volume restored by the addition of distilled water.

A part of the original stock solution, found by analysis to contain 5,760 parts per million of total solids, was diluted with an equal quantity of distilled water to obtain a culture solution of one-half that concentration, or 2,880 parts per million. Another part was diluted with 3 parts of distilled water to make a culture solution having 1,440 parts per million. These two diluted solutions were also tested electrically both in the natural condition and after boiling. The results of the electrical tests for all three culture solutions are shown in Table 1.

TABLE 1.—*Total solids, specific conductance, and alkalinity of the three culture solutions before and after boiling*

Solution No.	Total solids (parts per million)	Specific conductance at 25° C.		Alkalinity ( $P_H$ )	
		Before boiling	After boiling	Before boiling	After boiling
1.....	5,760	0.006873	0.007193	8.0	8.0
2.....	2,880	.003544	.003907	7.9	7.8
3.....	1,440	.002008	.002075	7.9	7.8

In the experiment of March, 1927, four lots of seedlings of 25 plants each of barley and of wheat were placed in jars of solution No. 3, containing 1,440 parts per million total solids. There were two lots each of barley and wheat in jars of solution No. 2, containing 2,880 parts per million of total solids, and three lots each of barley and wheat in jars of the original solution containing 5,760 parts per million. The seedlings were left in contact with the solution for 11 days, during which period a portion of it was absorbed by the roots and the water was transpired by the leaves. At the end of this period each jar was weighed, and the loss was taken as the measure of the quantity of the solution absorbed and of water transpired.

At the end of this first period also the seal of each jar was broken and a portion of the solution was withdrawn to be tested electrically for conductance and alkalinity, both in its natural condition and after boiling. After these tests the solution that had been withdrawn was then returned to the jar, and enough distilled water was added to replace the quantity that had been transpired. After this addition of distilled water another sample of the culture solution was withdrawn to be tested electrometrically as before. This tested portion was then returned to the culture jar, the jar was again sealed, and the seedlings were allowed to absorb the solution for a period of 13 days.

At the end of this second period of growth each solution jar was again weighed to determine the transpiration loss. The seedlings were then removed from the solution, and each lot was weighed in the fresh condition and again after drying to constant weight at 100° C. The solution remaining in each jar was sampled to be tested electrometrically, following which test each solution was again made up to its original volume by the addition of distilled water and again tested electrometrically. After this test each set of solutions of the same original concentration as used for each kind of seedlings were composited and samples were taken for analysis. In these analyses the six chief constituents were determined for each of the three solution concentrations as shown in Table 2.

TABLE 2.—Results of analyses of original culture solutions Nos. 1, 2, and 3 and of composites as restored to original volume at the conclusion of the experiment

[Data in parts per million]

Solids and ions identified	Solution No. 1			Solution No. 2			Solution No. 3		
	Original solution	After barley	After wheat	Original solution	After barley	After wheat	Original solution	After barley	After wheat
Total solids.....	5,760	5,240	5,280	2,880	2,472	2,456	1,440	1,128	1,200
Calcium.....	325	291	292	162	147	147	81	66	60
Magnesium.....	52	44	45	26	17	15	13	9	8
Bicarbonate.....	112	288	264	56	168	192	28	72	96
Chloride.....	588	616	598	294	280	256	147	112	112
Sulphate.....	2,688	2,542	2,545	1,344	1,224	1,205	672	574	570
Nitrate.....	296	144	160	133	0	0	66	0	0
Total ions identified....	4,031	3,925	3,894	2,015	1,836	1,825	1,007	833	846

Table 2 shows that the solutions when restored to original volume were slightly less concentrated at the end of the experiment than at the beginning. It must be realized, however, that the quantity of water absorbed by the seedlings during the 24 days of the experiment was approximately equal to the original volume in each case. This result makes it very evident that the plants did not absorb the solution as it existed in contact with their roots, but that they absorbed chiefly the water and only a small proportion of the dissolved material. In general, each of the ionic constituents was partially absorbed by the plants. The exception was the bicarbonate. With this constituent in every case the quantity identified at the end of the experiment was greater than at the beginning. This increase was probably due to the evolution of carbon dioxide from the plant

roots. The effect of this process was also indicated by a comparison of the  $P_H$  values of the solutions at the beginning and at the end of the experiment. The average  $P_H$  value of the original solutions was 7.9, whereas at the end it had dropped to 7.6 for the barley solutions and 7.5 for the wheat solutions. This increase in the bicarbonate anion was partly offset by decreases in the sulphate and nitrate anions, both of which were taken up to a greater extent than the chloride anion. The nitrate was completely absorbed from the more dilute solutions.

#### INTERPRETATION OF RESULTS

The primary aim of this experiment was to observe the effect of the absorption by the plants upon the concentration of the solution. In order to avoid unnecessary complications, it seemed desirable not to use up any of the solution for analysis during the progress of the experiment. Yet it was important to compare the solution concentrations at the end of each growth period with the initial concentrations. For this purpose it seemed practicable to use the method of electrical conductance. With this method a sample of the solution could be withdrawn from each culture jar, its conductance measured, and the sample returned to the jar without appreciable alteration.

In order to standardize these conductance determinations for each solution, each sample that was analyzed gravimetrically for total solids at the beginning and at the end of the experiment was also tested electrometrically. For example, solution No. 1 at the beginning of the experiment showed by evaporation 5,760 mgm. per liter of total solids. The same sample showed a specific conductance at 25° C. of 0.006873 reciprocal ohm. Whence:  $\frac{0.5760}{0.006873} = 83.8$ , or  $83.8 \times 0.006873 = 0.5760$ . At the conclusion of the experiment the three solutions of No. 1 in which barley seedlings had been grown were restored to their original volume by the addition of distilled water and tested for conductance. The mean of these three observations gave a specific conductance at 25° C. of 0.006653 reciprocal ohm. These three solutions were then composited and a sample taken for analysis, which showed total solids of 5,240 mgm. per liter. Whence:  $\frac{0.5240}{0.006653} = 78.7$ . The mean of these two factors, i. e., 81.3, was taken as the conversion factor for interpreting the conductance determinations made on cultures of barley in solution No. 1 during the progress of the experiment. By the same method conversion factors were obtained for each of the other culture solutions.

The detailed interpretation of the observations made during the experiment may be followed through by reference to Table 3, which gives the results obtained from three lots each of barley and wheat seedlings grown in solution No. 1. The volume of solution used for each culture was 950 c. c., and this contained originally 5,472 mgm. of salts or total solids.

TABLE 3.—Results of growing barley and wheat seedlings in salt solution No. 1

[25 plants in 950 c. c. culture solution containing 5,472 mgm. of salt]

Growth period and item	Barley seedlings			Wheat seedlings		
	Lot No. 10	Lot No. 11	Lot No. 12	Lot No. 10	Lot No. 11	Lot No. 12
First growth period, 11 days.						
Water transpired..... c. c.	232	215	228	241	263	332
Salts...mgm. { Residual <sup>a</sup>	5,105	5,165	5,144	5,159	5,219	5,107
{ Restored <sup>b</sup>	5,232	5,269	5,246	5,421	5,249	5,202
Mean.....	5,168	5,217	5,195	5,290	5,234	5,154
Salts absorbed.....mgm.	304	255	277	182	238	318
Concentration of original solution.....ppm. <sup>c</sup>	5,760	5,760	5,760	5,760	5,760	5,760
Concentration of absorbed solution.....do.	1,310	1,177	1,215	755	905	958
Second growth period, 13 days:						
Original salt content.....mgm.	5,168	5,217	5,195	5,290	5,234	5,154
Water transpired.....c. c.	468	434	406	596	575	639
Salts...mgm. { Residual <sup>a</sup>	4,890	4,906	4,779	5,059	4,904	4,553
{ Restored <sup>b</sup>	5,073	5,168	5,173	5,059	5,127	5,160
Mean.....	4,981	5,037	4,976	5,059	5,015	4,856
Salts absorbed.....mgm.	187	180	219	231	219	298
Concentration of original solution.....ppm. <sup>c</sup>	5,440	5,492	5,468	5,588	5,509	5,425
Concentration of absorbed solution.....do.	401	415	539	387	381	466
Total growth period, 24 days:						
Total water transpired.....c. c.	698	649	634	837	838	971
Total salts absorbed.....mgm.	491	435	496	413	457	616
Concentration of absorbed solution.....ppm. <sup>c</sup>	703	670	752	493	545	634
Dry weight of 25 plants.....gm.	2.47	2.14	2.23	2.44	2.77	2.85

<sup>a</sup> Computed from conductance of residual solutions.<sup>b</sup> Computed from conductance of restored solutions.<sup>c</sup> Parts per million.

During the first 11 days of the growth period the barley plants of lot No. 10 transpired 232 c. c. of water, as determined by weighing the culture jar at the beginning and at the end of that growth period. There remained, therefore, 718 c. c. of solution in the culture jar. A sample of this residual solution when tested showed a specific conductance at 25° C. of 0.008747 reciprocal ohm. This value multiplied by the conversion factor 81.3 and the product multiplied by the volume of the residual solution, 718, gives a figure for the quantity of salt left in the solution, viz, 5,105 mgm.

The sample of the residual solution that was tested for conductance was then returned to its culture jar, and distilled water was added to restore the solution to its original volume. After this restoration, another sample was taken for a conductance determination. This sample showed a specific conductance at 25° C. of 0.006774 reciprocal ohm. This figure when multiplied by the conversion factor 81.3 and the product multiplied by the solution volume, 950, gives 5,232 as another figure for the quantity in milligrams of salt in the culture solution. It was deemed advisable to make these two conductance determinations partly as a check against errors of observation and partly because it was thought that the culture solution might be near the saturation point with respect to one or more of its constituent salts, and if this were true the absorption of water by the plants might result in the precipitation of a portion of the dissolved material in the residual solution. The mean of these two results was taken as the best estimate of the quantity of salt remaining in the solution at the end of the first growth period.

The quantity of salt originally in the solution was known to be 5,472 mgm. From this was subtracted the quantity estimated to be in the residual solution, giving a difference of 304 mgm. as the quantity of salt absorbed by the plants. This quantity of salt in relation to the quantity of water transpired, 232 c. c., gives 1,310 mgm. per liter, for the concentration of the absorbed solution. For convenient comparison, this figure is placed in the table in close proximity to that for the concentration of the original solution.

After determining the conductance of the restored solution at the end of the first growth period, the jars were resealed for the second growth period of 13 days. For lot No. 10 the figure for the original salt content for this period was taken as the same as at the end of the first period, 5,168 mgm. During the second period the plants transpired 466 c. c. of water. At the conclusion of this period the quantity of salt remaining in the culture solution was again determined by two conductance readings, and the mean of these was taken as representing the final salt content of the solution. By subtracting this figure from that for the original salt content the quantity absorbed by the plants was obtained, and from this and the quantity of water transpired the concentration of the absorbed solution was computed. Finally the quantity of water transpired for the whole period of growth is given, together with the quantity of salt absorbed, and from these two is computed the concentration of the absorbed solution.

TABLE 4.—Results of growing barley and wheat seedlings in salt solution No. 2

[25 plants in 950 c. c. culture solution containing 2,736 mgm. of salt]

Growth period and item	Barley seedlings		Wheat seedlings	
	Lot No. 8	Lot No. 9	Lot No. 8	Lot No. 9
First growth period, 11 days:				
Water transpired.....c. c.	256	288	328	340
Salts.....mgm. {Residual <sup>a</sup> .....	2,524	1,853	2,407	2,516
{Restored <sup>c</sup> .....	2,746	2,579	2,459	2,524
Mean.....	2,524	2,580	2,433	2,520
Salts absorbed.....mgm.	212	156	303	216
Concentration of original solution.....ppm <sup>d</sup> .....	2,880	2,880	2,880	2,880
Concentration of absorbed solution.....ppm <sup>d</sup> .....	828	542	924	635
Second growth period, 13 days:				
Original salt content.....mgm.	2,524	2,580	2,433	2,520
Water transpired.....c. c.	578	653	712	747
Salts.....mgm. {Residual <sup>a</sup> .....	2,253	2,249	2,252	.....
{Restored <sup>c</sup> .....	2,284	2,369	2,278	2,444
Mean.....	2,268	2,309	2,265	2,444
Salts absorbed.....mgm.	256	271	168	76
Concentration of original solution.....ppm <sup>d</sup> .....	2,637	2,716	2,561	2,633
Concentration of absorbed solution.....ppm <sup>d</sup> .....	443	415	236	102
Total growth period, 24 days:				
Total water transpired.....c. c.	834	941	1,040	1,087
Total salts absorbed.....mgm.	468	427	471	292
Concentration of absorbed solution.....ppm <sup>d</sup> .....	561	454	453	269
Dry weight of 25 plants.....gm.	2.34	2.65	2.70	2.53

<sup>a</sup> Computed from conductance of residual solutions.<sup>b</sup> Only one observation used.<sup>c</sup> Computed from conductance of restored solutions.<sup>d</sup> Parts per million.

Besides the three lots of barley seedlings grown in solution No. 1, with the results shown in Table 3, three lots of wheat seedlings also were grown in cultures of the same solution concentration, and the results for these are also given in Table 3. Two lots of barley were grown in solution No. 2, containing 2,880 mgm. of salts per liter, for which the results are given in Table 4. With the same solution there were two lots of wheat seedlings, the results from which are also shown in Table 4. Solution No. 3, made by dilution of No. 1 with three parts of distilled water, was used for four lots of barley and four of wheat, the results for which are given in Table 5.

TABLE 5.—Results of growing barley and wheat seedlings in salt solution No. 3

[25 plants in 950 c c culture solution containing 1,368 mgm of salt]

Growth period and item	Barley seedlings				Wheat seedlings			
	Lot No. 4	Lot No. 5	Lot No. 6	Lot No. 7	Lot No. 4	Lot No. 5	Lot No. 6	Lot No. 1
First growth period, 11 days								
Water transpired.....c. c.	408	341	267	351	340	337	393	305
Salts.....mgm.								
{Residual a.....	1,131	1,219	1,329	1,122	1,253	1,194	1,223	1,276
{Restored b.....	1,305	1,228	1,263	1,279	1,257	1,207	1,271	1,236
Mean.....	1,218	1,223	1,296	1,200	1,255	1,200	1,247	1,256
Salts absorbed.....mgm.	150	145	72	168	113	168	121	112
Concentration of original solution.....ppm c.	1,440	1,440	1,440	1,440	1,440	1,440	1,440	1,440
Concentration of absorbed solution.....ppm c.	368	425	269	479	333	498	308	367
Second growth period, 13 days								
Original salt content.....mgm.	1,218	1,223	1,296	1,200	1,255	1,200	1,247	1,256
Water transpired.....c. c.	671	385	596	681	750	620	723	717
Salts.....mgm.								
{Residual a.....	956	1,155	957	995	1,068	1,198	1,119	1,209
{Restored b.....	1,011	1,110	1,093	1,038	1,096	1,097	1,148	1,184
Mean.....	983	1,132	1,025	1,016	1,082	1,097	1,133	1,196
Salts absorbed.....mgm.	235	91	271	184	173	103	114	60
Concentration of original solution.....ppm c.	1,282	1,287	1,364	1,263	1,356	1,263	1,313	1,322
Concentration of absorbed solution.....ppm c.	350	236	455	270	231	166	158	84
Total growth period, 24 days:								
Total water transpired.....c. c.	1,079	726	863	1,032	1,090	957	1,116	1,022
Total salts absorbed.....mgm.	385	236	343	352	286	271	235	172
Concentration of absorbed solution.....ppm c.	356	325	397	341	262	283	211	168
Dry weight of 25 plants.....gm.	2.80	1.94	2.36	2.65	2.55	2.42	2.62	2.21

a Computed from conductance of residual solutions.

b Computed from conductance of restored solutions.

c Parts per million.

d Only one determination used.

Tables 3 to 5, inclusive, each include the results for individual lots of seedlings, and it is to be expected that they would show a certain diversity, even though the conditions of the experiment were made as uniform as practicable. In order to summarize these results and also to facilitate comparison between the results obtained from the barley and the wheat seedlings when grown in solutions of the same concentrations, the results from comparable individual lots have been averaged and are shown for solution No. 1 in Table 6. This table includes, in addition to the averages of the quantity and concentration of the absorbed solution, a comparison of the average quantity of salts absorbed as determined by conductance and by gravimetric measurements. This comparison gives some indication as to the degree of confidence that may be placed in the final results. A similar comparison of averages for the barley and wheat seedlings

grown in solution Nos. 2, and 3 is also given. These comparisons between barley and wheat show certain differences that may be noted, but probably should not be greatly stressed because the numbers of cultures involved are not large and the differences may not be significant. However, it may be seen that in all three comparisons the wheat seedlings transpired slightly more water than the barley seedlings and also that they produced slightly more dry matter. There are not, however, any consistent differences between the two classes of seedlings with respect to the quantities of salt absorbed.

TABLE 6.—Average results obtained with separate lots of barley and wheat seedlings grown in culture solutions Nos. 1, 2, and 3, having initial concentrations respectively of 5,760, 2,880, and 1,440 parts per million

Growth period and item	Solution No. 1 (3 lots averaged)		Solution No. 2 (2 lots averaged)		Solution No. 3 (4 lots averaged)	
	Barley	Wheat	Barley	Wheat	Barley	Wheat
First growth period, 11 days:						
Solution absorbed.....c. c.	225	279	272	334	342	344
Concentration of absorbed solution.....ppm. °	1,234	873	685	779	385	376
Second growth period, 13 days:						
Solution absorbed.....c. c.	435	603	615	729	583	702
Concentration of absorbed solution.....ppm. °	452	411	429	169	328	160
Total growth period 24 days:						
Solution absorbed.....c. c.	660	882	887	1,063	925	1,046
Concentration of absorbed solution.....ppm. °	718	557	507	361	355	231
Salts absorbed, conductance.....mgm.	474	495	447	381	329	241
Salts absorbed, gravimetric.....do.	494	456	388	403	296	228
Dry weight of 25 plants.....gm.	2.28	2.69	2.49	2.76	2.44	2.45

° Parts per million.

Another comparison of the results is shown in Table 7, where the averages for the three sets of barley seedlings are given. Each lot of barley was grown in a solution of different concentration. It will be observed that with a single exception—solution No. 2 for the second growth period—the barley plants absorbed less water from the more concentrated solutions. Also, the concentration of the absorbed solution increased with the concentration of the culture solution in all cases. Correspondingly, the quantity of salt absorbed by the plants increased with the concentration of the solutions.

TABLE 7.—Average results obtained from growing barley and wheat seedlings in three different solutions, Nos. 3, 2, and 1, having concentrations, respectively, of 1,440, 2,880, and 5,760 parts per million

Growth period and item	Barley seedlings			Wheat seedlings		
	Solution No. 3	Solution No. 2	Solution No. 1	Solution No. 3	Solution No. 2	Solution No. 1
First growth period, 11 days:						
Solution absorbed.....c. c.	342	272	225	344	334	279
Concentration of absorbed solution .ppm. °	385	685	1,234	376	779	873
Second growth period, 13 days:						
Solution absorbed.....c. c.	583	615	435	702	729	603
Concentration of absorbed solution .ppm. °	328	429	452	160	169	411
Total growth period, 24 days:						
Solution absorbed.....c. c.	925	887	660	1,046	1,063	882
Concentration of absorbed solution .ppm. °	355	507	718	231	361	557
Salts absorbed, conductance.....mgm.	329	447	474	241	381	495
Salts absorbed, gravimetric.....do.	296	388	494	228	403	456
Dry weight of 25 plants.....gm.	2.44	2.49	2.28	2.45	2.76	2.69

° Parts per million.

A similar comparison is made in Table 7 for the three sets of wheat seedlings. In this series there are two exceptions to the rule that more water was absorbed from the more dilute solutions. With but one exception the concentration of the absorbed solution was higher with the more concentrated solutions and also more salt was absorbed by the plants from the more concentrated solutions. There appears not to have been any correlation between the dry weight of the plants and the solution conditions, perhaps because the range of solution concentrations was well within the range to which these plants are able to adapt themselves.

#### RANGE OF SOLUTION CONCENTRATIONS

In planning this series of experiments it was aimed to keep the concentrations of the solutions well within the limits which occur commonly in the root zone of irrigated soils. It must be kept in mind that the concentration of the soil solution is computed from factors that are different from those used in computing the salt content of the soil. In the latter computation the quantity of soluble material is referred to the dry weight of the soil. Thus when a soil is said to contain 1,440 parts per million of soluble salts, the implication is that for each million pounds of dry soil there are 1,440 pounds of salt. The percentage of water is also usually referred to the dry soil. Therefore, if a soil has a field-carrying capacity for water of 25 per cent, the relationship is such that for each 100 pounds of soil (dry weight) there is held in suspension 25 pounds of water. If in such a soil it were found that each 100 pounds of dry soil contained 0.144 pound of soluble salts, it is assumed that this salt would all be dissolved in the water held in the soil; so that the concentration of the soil solution would be said to be four times that of the salt content of the soil, or 5,760 parts per million.

From this explanation it will be evident that the highest solution concentration developed in these experiments, i. e., at the end of the second growth period of the wheat seedlings in lot No. 12, when the concentration was 15,614 parts per million, or about 1.56 per cent, was not higher than often occurs in the solution of an irrigated soil. A similar solution concentration might be assumed to exist in a soil containing 0.2 per cent of readily soluble salts and about 14 per cent of water. On the other hand, the lowest solution concentration occurring in this experiment, i. e., about 1,300 parts per million, is probably rather higher than that existing in a soil that has been heavily irrigated or saturated with rain.

#### DISCUSSION OF RESULTS

The results of these experiments appear to warrant the conclusion that crop plants do not absorb water and dissolved substances from the soil solution in the same proportions that these constituents occur together in that solution. With the range of solution concentrations used, and under the conditions of the experiments here described, the residual solutions were definitely more concentrated than the original solutions. The quantity of water absorbed by the plants during the 24 days of the experiments was approximately equivalent to the original volume of the culture solutions, yet at the end of that period

the residual solutions contained 78 to 91 per cent of the salts originally present.

It is true that these experiments were conducted in a laboratory and not in the field, and that plants were grown in culture solutions and not in soil; yet it is believed that the results are fairly applicable to field conditions. Furthermore, there is abundant field evidence to show that when saline irrigation water is used so sparingly that the root zone is seldom or never leached, it is merely a question of time until the soil solution of the root zone becomes so concentrated that crop plants can not absorb from it the water required to make normal growth.

Irrigators are sometimes inclined to believe that if it is necessary to use salty irrigation water it should be used sparingly, so as to avoid carrying to the field more salt than is necessary. In the light of such evidence as is available, this method of procedure is not to be recommended. The only known way to remove soluble salt from the soil of the root zone is by leaching. Consequently, if saline water must be used for irrigation, it should be applied in sufficient quantities not only to supply crop needs and to meet evaporation losses but also to leach the root zone and thus carry away the salts that are left by the water that is evaporated from the soil and absorbed by the plants. In other words, the more salt there is in irrigation water the more water should be used in irrigating. Most of the ordinary crop plants are not able to absorb the water needed for normal growth from a soil solution containing more than 1.5 to 2 per cent of salts. This means that if the irrigation water contains as much as 1,500 to 2,000 parts per million of salt, a sufficient quantity of it should be used so that at least 10 per cent of the quantity applied to the surface of the soil will percolate down through the root zone.

In actual irrigation practice with such water it would be necessary to use more than would be required to insure an average of 10 per cent percolation. The reason for this is that the soil of the root zone of a whole field is seldom uniform in texture or permeability. If only 10 per cent of the water applied were to percolate through the root zone, there would be spots in the field where much less than 10 per cent would percolate; and in those spots there would be grave danger of accumulating excessive concentrations in the soil solution. In order to insure effective percolation and consequent leaching of the root zone, it would be necessary to follow one of two courses—either to apply enough water to the whole field to insure leaching in the areas of least permeable soil, or by a system of interior borders provide a means of holding the water longer on the less permeable areas.

It is not necessary, of course, to use water enough to leach the root zone at every irrigation, particularly where the water is not very salty or when the salts in solution are in part salts of low solubility. With many irrigation waters as much as 60 to 80 per cent of the total salt content consists of salts of such low solubility that they are precipitated from the soil solution before injurious concentrations are reached. This fact needs to be taken into account in estimating what proportion of the irrigation water should be forced through the root zone.

It will be readily understood that in order to leach the root zone the conditions of the subsoil below it must be such that the percolating water may pass away through either natural or artificial

channels. Soils that are underlain by strata of impermeable material such as hardpan, rock, or tight clay, or below which the subsoil is already saturated with stagnant water, can not be leached and consequently are almost certain to become unproductive if irrigated with saline water. This fact makes it essential to take into account the subsoil conditions of irrigable land if the plans for its utilization contemplate its long-continued productivity.

It is coming to be generally recognized that irrigated lands need to have an assured and effective system of drainage, either natural or artificial. Unfortunately, it is not yet so generally recognized that the real function of a drainage system is quite as much to remove surplus salt as to remove surplus water. It is now regarded as normal and routine engineering practice to measure the quantity of water that is delivered to the land for irrigation or that is discharged from irrigated land as drainage, but there are as yet relatively few irrigation engineers who appreciate the need of, or who are equipped to make, determinations of the salt content of irrigation water or of the drainage. With respect to any area of irrigated land there should be available, not only to the farmer but also to the investor, accurate information as to the quantity and character of the salts that are being carried to the land by the irrigation water and also as to the ultimate disposition that is being made of this salt. If the conditions of the subsoil or the methods of irrigation are such that the salt is largely remaining in the root zone, then it is inevitable that the land must ultimately become unproductive. On the other hand, if it can be shown that drainage conditions are such that the salts of high solubility brought in by the irrigation water are being carried away in the drainage, there can be reasonable assurance that the irrigated lands should long continue to be productive.

# EXPERIMENTS WITH SODIUM CHLORATE AND OTHER CHEMICALS AS HERBICIDES FOR FIELD BINDWEED<sup>1</sup>

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## INTRODUCTION

Field bindweed (*Convolvulus arvensis*) is regarded as the most noxious weed in several of the Western States and is widely distributed in Kansas. In some cases infested farms are reduced in value more than one-half and loan companies often refuse to accept them as security.

Considering the seriousness of the situation and the fact that methods now in use (5)<sup>3</sup> in Kansas are not wholly satisfactory, further experimental work seemed desirable. The use of chemicals as herbicidal agents is not new, but so far as is known to the authors sodium chlorate had not been tried in any experiments with bindweed previous to those herein reported.

## REVIEW OF LITERATURE

A considerable amount of work has been done by various investigators relating to the eradication of bindweed. Intensive cultivation has proved effective in several States as shown by the following workers: Call and Getty (5) in Kansas; Barnum (3) and Bioletti (4) in California; Cox (6) of the United States Department of Agriculture; Stewart and Pittman (13) in Utah; and in Idaho (10), as reported by the director of the Idaho Experiment Station.

Chemicals have been used more or less generally. Krauss (12) and Wilcox (15) found sodium arsenite effective. Gray (7) also found it effective within the fog belt of California, but not nearly so successful (8) in the less humid section of the State. This latter finding was verified by Barnum (3). In Idaho (10) carbon bisulphide is recommended for use on small areas, but the cost prohibits its general use. Barnum (3) in California arrived at essentially the same conclusion. Call and Getty (5) and Barnum (3) found that salt will kill this weed, but that it has the disadvantage of preventing the growth of all vegetation for an indefinite period. Åslander (1) found dilute sulphuric acid of value as a herbicide. The same author (2) has recently reported good results with chlorates in the control of Canada thistle. Hutcheson and Wolfe (9) found sodium arsenite, nitrate of soda, and common salt to be effective under certain conditions in eradicating hawkweed. Thompson and Robbins (14) report results with the use of 37 different chemicals including inorganic

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<sup>2</sup> The cross sections and slides used in making Figures 1 and 2 were prepared by E. C. Miller, professor of plant physiology. The drawings were made by S. Fred Prince, biological artist. The soil samples for nitrification studies were collected and incubated by P. L. Gainey, professor of soil bacteriology, and the sodium arsenite used was furnished through the courtesy of Edgar M. Ledyard, agricultural director of the United States Smelting, Refining, and Mining Co., Salt Lake City, Utah.

<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 767.

salts, acids and acid formers, gas-forming substances, and oils and other organic substances. Kitchin (11) found zinc sulphate and copper sulphate effective in the control of weeds in pine seed beds.

#### PREVIOUS EXPERIMENTS IN KANSAS

Bindweed was recognized as a serious pest in Kansas as early as 1908, and experiments looking to its control were begun at the Dodge City branch station as early as 1913. These experiments consisted in smothering with a mulch, plowing, hoeing, pasturing with hogs, the use of smother crops, and salting. Salting proved to be the only effective treatment.

In 1915 an infested area at the branch station at Hays, Kans., was fenced and pastured with sheep, but the sheep had to be forced by hunger to graze closely enough to weaken the plants, and the results were so unsatisfactory as to make the method impractical. In 1919 experiments were begun which included different rates of salting, application of fuel oil, intensive fallow, fallow in rotation with smother crops and smother crops alone. Intensive fallow with or without smother crops on large areas and salting the small patches at the rate of 1 pound of salt to the square foot were found to be the only effective methods.

#### EXPERIMENTAL METHODS

The experiments herein reported were begun in August, 1925, at the Kansas Agricultural Experiment Station, Manhattan, Kans., in a field on which a uniform and thick stand of bindweed had been growing for several years. The soil is a silt loam of the Oswego series, uniform in fertility, and nearly level in topography. This field has been under cultivation for 50 years or more and until 1924 had been used for experimental work in farm crops.

Twelve plots, each 20.87 feet square, or approximately one one-hundredth acre in area, were located in the center of the most vigorous and uniform bindweed growth. They were marked by permanent iron posts driven deep in the ground at each corner. A 2-foot alley was left between the plots. In order to obtain a definite record of the condition of each plot with reference to the stand of bindweed, quadrats 3 by 3 feet were located in each and permanently marked by iron posts. A frame 3 by 3 feet inside dimensions and cross sectioned into square-foot areas was used in obtaining accurate counts of the number of weeds growing in the quadrat.

It was found that some caution is necessary in determining the effect of any treatment on small plots, because of plants sending out underground stems from adjoining plots and alleys. Thus in certain plots in which all plants had been killed a few stray ones were found which had crept in from the adjoining untreated areas. Examination soon disclosed their source and these areas were treated to prevent further intrusion.

It was found by trial that 1 gallon of the solution used was sufficient to moisten the leaves of the plants on a one one-hundredth-acre plot. Since nothing definite was known to begin with as to the concentration and frequency of spraying necessary to kill bindweed, these factors were determined more or less arbitrarily. In deciding

upon the frequency of treatment the growth of the bindweed was taken into consideration. Usually the weeds were treated when they had attained a height of from 3 to 5 inches. In a few cases the cultivation of the fallow plots was delayed by rain. The frequency of treatment is indicated in the tables which follow. The chemicals were applied by means of a 3-gallon knapsack compressed air sprayer.

In order to determine the effect of the various treatments the plants in each quadrat were counted just before each treatment.

#### EXPERIMENTS WITH DIFFERENT CHEMICALS

The strength of the solution, the time of treatment, and the number of bindweed plants per quadrat before each treatment are shown in Table 1. Except as otherwise noted each plot was treated every time a count of bindweed was made. Thus the time and frequency of treatment indicated in the table by the date on which counts were made. The treatment of the fallow plots consisted of cultivation, and it is, of course, understood that the no-treatment or control plots were not treated in any way. The number of plants, however, was determined on the control plots whenever they were counted on the other plots.

TABLE 1.—*Number of bindweed plants per quadrat in fallow and untreated plots and in plots treated with different chemicals; August 18, 1925, to May 1, 1927*

Date of treatment	Number of bindweed plants per quadrat											
	Plot 1; copper sulphate, 12.5 per cent	Plot 2; fallow	Plot 3; zinc chloride, 12.5 per cent	Plot 4; sodium arsenite, 2 per cent	Plot 5; no treatment	Plot 6; sodium chlorate, 25 per cent	Plot 7; sodium chlorate, 12.5 per cent	Plot 8; fallow	Plot 9; sodium arsenite, 1 per cent	Plot 10; zinc chloride, 6.25 per cent	Plot 11; no treatment	Plot 12; copper sulphate, 6.25 per cent
1925												
Aug. 19.....	31	27	44	25	22	27	35	25	43	65	33	41
Sept. 2.....	25	25	35	23	23	23	24	35	28	45	30	27
Sept. 16.....	40	42	47	38	17	19	40	58	30	47	28	29
Sept. 29.....	54	56	56	47	23	4	4	36	46	59	32	31
1926												
Apr. 30.....	70	77	67	<sup>b</sup> 65	49	0	0	50	<sup>b</sup> 73	79	101	45
May 11.....	67	75	77	77	—	0	0	49	81	112	—	60
June 2.....	69	91	93	88	—	0	0	49	84	98	—	60
June 14.....	67	91	71	66	—	0	0	51	61	90	—	50
June 30.....	—	88	—	—	—	48	—	—	—	—	—	—
July 12.....	75	76	66	58	—	0	0	48	54	71	—	40
July 26.....	—	62	—	—	—	30	—	—	—	—	—	—
Aug. 8.....	58	44	47	29	—	23	—	23	—	—	—	45
Aug. 17.....	—	89	—	—	—	0	0	30	38	43	—	—
Aug. 30.....	—	123	—	—	—	34	—	—	—	—	—	—
Sept. 18.....	57	118	32	20	—	0	0	31	42	52	—	59
Oct. 19.....	75	98	46	24	—	0	0	22	59	58	—	62
1927												
Apr. 30.....	68	76	61	35	—	0	0	30	46	49	—	9

<sup>a</sup> Plots 6 and 7 were not treated after Sept. 16, 1925.

<sup>b</sup> In 1926 the strength of spray on plots 4 and 9 was increased to 4 per cent and 2 per cent, respectively.

It will be noted that none of the treatments except that with sodium chlorate was effective in decreasing the number of bindweed plants. On the contrary there seems to have been a marked increase in numbers in some cases. Sodium arsenite and zinc chloride killed the tops of the plants and at first appeared to be very effective, but

new growth appeared soon after each treatment and the roots seemed to be uninjured. Copper sulphate had little effect except the fourth application, which killed almost all top growth. This application was made on September 29 and may have coincided with a critical period in the life of the plants. This result suggests the need of more information regarding the relation between stage of growth and susceptibility. In all cases except possibly the sodium arsenite, the weaker solutions seemed to have been about as effective as the stronger. At the suggestion of the manufacturers the strength of the solution of this chemical was increased in 1926. The effectiveness of the treatment was somewhat increased, but it did not approach the sodium chlorate in this respect. It will be noted that it was necessary to cultivate the fallow plots more frequently than it was necessary to spray the chemically treated plots.

Sodium chlorate was markedly and quickly effective, only three applications being necessary to kill all the plants. The effect on the appearance of the plants was also very different from that produced by the other herbicides. Its action at first appeared to be slow. Comparatively little effect was noticed at first, but gradually the plants became pale green or yellow, the leaves began to curl, and finally the entire plant withered and died. Each succeeding day showed more and more dead and dying plants. The older leaves died first, the reaction progressing toward the tip of the vines. After the second application it was observed that the vines were slender and assumed an upright position instead of the normal vigorous and prostrate growth.

On September 29—that is, at the time of the fourth application for other treatments—the plants on the sodium chlorate plots had been so reduced in number and vigor that further treatment was considered unnecessary. As a matter of fact it is doubtful if the third treatment on September 16 was necessary, since the plants at that time were obviously weak and were rapidly dying. On September 29 the roots of the bindweed plants on the various plots were examined under the microscope. Those from the plots treated with sodium chlorate had a dark ring around the wood portion which was not observed in those from other plots. There was also a noticeable absence of new shoots on the roots. These conditions prompted a more detailed microscopic and chemical examination which was made at the time the growth ceased in the fall.

On September 30, twenty or more plants in the sodium chlorate plots which because of special vigor had previously been marked for observation, were found to be making no growth but were turning yellow and becoming more sickly in appearance. On October 5, approximately three weeks after the last treatment, no increase in growth was apparent, the plants being dead or dying. New plants reappeared from time to time, only to fade away in the course of four or five days.

A very heavy white frost occurred on October 10, the minimum temperature reaching 25° F. Observations were made as soon as the frost had disappeared and again three days later. Bindweeds surrounding the experimental plots were damaged very little, if any. This was also true of all the experimental plots except those which had been treated with sodium chlorate. These, however, had been

severely injured, most of them being frozen to the ground. This observation seems to be especially pertinent in view of the fact that untreated plots were later observed to have survived temperatures of 14° F. and the tops were not killed until late in November. This would seem to indicate that bindweed is not sensitive to frost as is commonly believed.

#### ROOT STUDIES

The gradual killing of the plants by sodium chlorate and their susceptibility to freezing, as previously noted, suggested a study of the roots. Accordingly, on October 15, roots were taken from the various plots and cross-sections were made and examined. Important differences were noted in the starch content of the cells and in the condition of the cell walls. The cells in the roots from the untreated plots were filled with starch granules and the cell walls were intact. On the other hand, roots from the plots treated with sodium chlorate were characterized by the absence of starch granules, and the cell walls in many cases showed unmistakable signs of disintegration. An attempt is made to show this condition in Figures 1 and 2, which are camera-lucida drawings of cross sections of the roots from the various plots. The heavily shaded areas within the cells represent stored starch. The light shading between cells represents disintegration of cell walls.

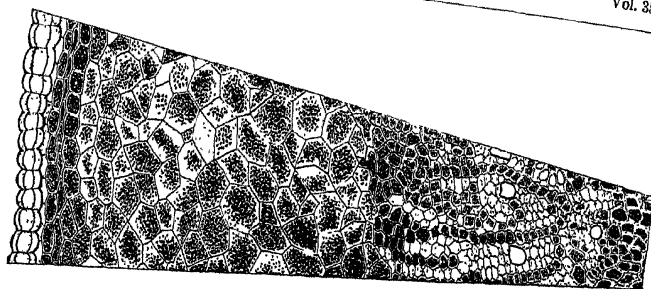
It will be noted that the condition of the roots as represented in the figures corresponds closely with the growth and condition of the plants as previously discussed.

Just why sodium chlorate affects the plants as it does is not known. Observations indicate that it interferes with photosynthesis and compels the plant to draw upon the food reserve in the roots, until the supply is exhausted and death occurs.

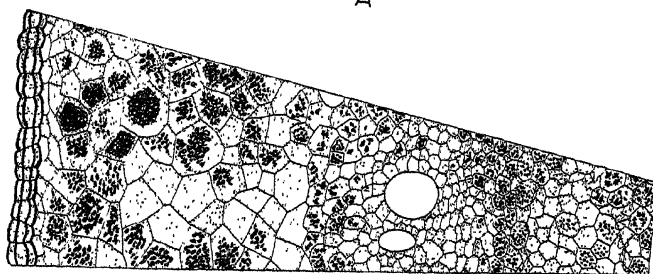
#### STRENGTH OF SODIUM CHLORATE SOLUTION AND FREQUENCY OF APPLICATION

The fact that the 12.5 per cent solution of sodium chlorate was as effective as the 25 per cent solution suggested the possibility of using even weaker solutions. It was also thought that the bindweed plants might be more susceptible at certain periods of growth and that the time of treatment might be an important factor. Experiments were therefore started in 1926 to determine the best time for treatment, the minimum number of treatments, and the minimum strength of solution that could be used effectively. Eleven additional plots were laid out and treated as indicated in Table 2. As in the preceding experiments the number of bindweed plants was determined just before each treatment, except as otherwise stated. The results are presented in Table 2.

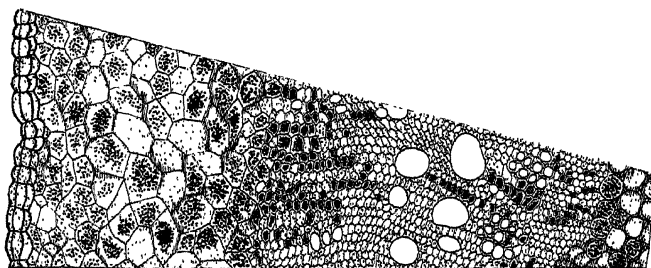
In general the treatments in 1926 seemed to be not quite as effective as those of the preceding year, owing it is believed to a severe drought, which retarded the growth of the bindweed and hence afforded less opportunity for exhaustion of the root reserves, which, as previously explained, seems to be a significant factor in control with sodium chlorate. Nevertheless the results in a broad way agree with those of the preceding year and afford additional proof of the effectiveness



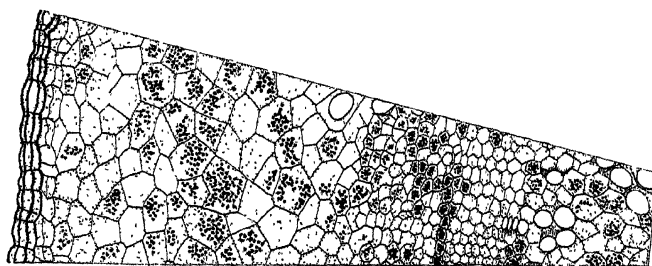
A



B



C



D

FIG. 1.—Cross sections of roots of bindweed from plots receiving: A, no treatment (control); B, copper sulphate treatment; C, fallow treatment; and D, zinc chloride treatment

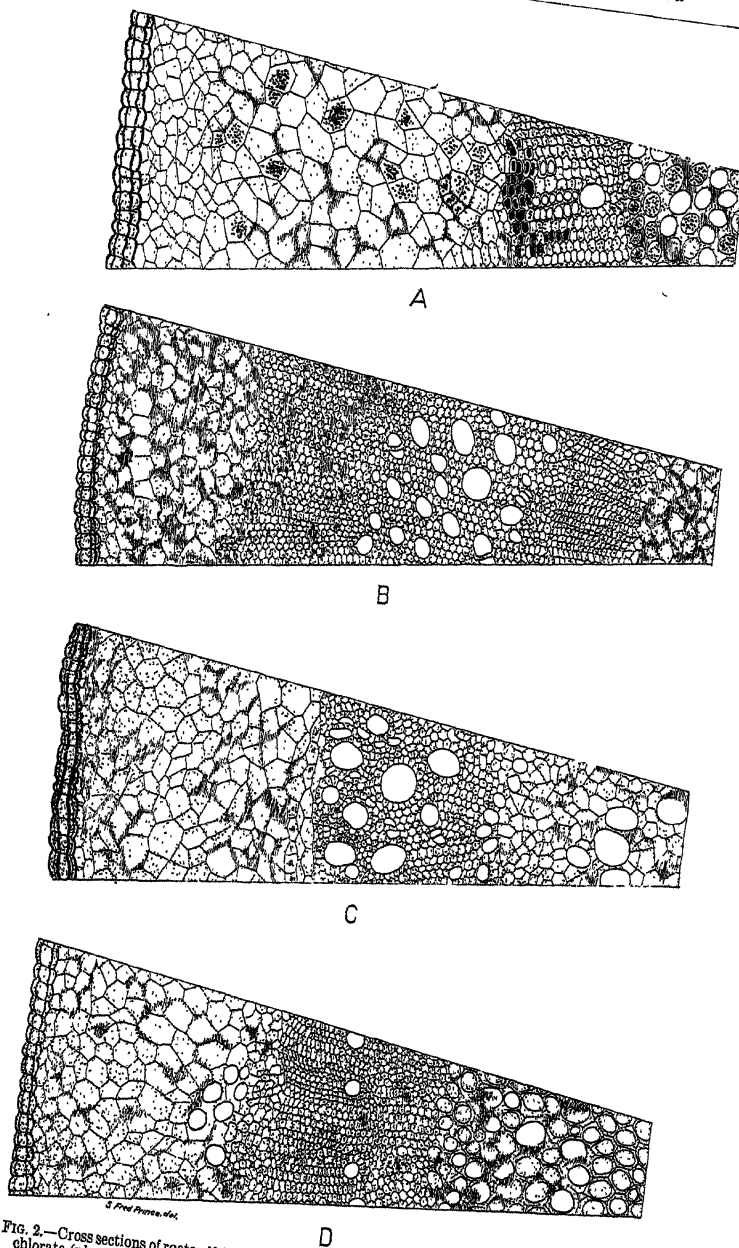


FIG. 2.—Cross sections of roots of bindweed from plots treated with: A, sodium arsenite; B, sodium chlorate (plant with top dead to crown); C, sodium chlorate (plant with top frosted to crown); and D, sodium chlorate (plant with top green)

of sodium chlorate. They also afford information as to the proper time and frequency of treatment.

TABLE 2.—Number of bindweed plants per quadrat in plots treated on different dates with sodium chlorate solution of different strengths

Date of treatment	Number of bindweed plants per quadrat										
	Plot 5, 12.5 per cent solu- tion	Plot 11, 12.5 per cent solu- tion	Plot 17, 6.25 per cent solu- tion	Plot 20, 3.125 per cent solu- tion	Plot 21, 12.5 per cent solu- tion	Plot 22, 12.5 per cent solu- tion	Plot 23, 12.5 per cent solu- tion	Plot 24, 12.5 per cent solu- tion	Plot 25, 12.5 per cent solu- tion	Plot 26, 12.5 per cent solu- tion	Plot 27, 12.5 per cent solu- tion
1926											
Apr. 30			28	73							
May 14			59	63	29	43	65				
June 2			57	48				63			
June 14			60	50			<sup>a</sup> 36	<sup>a</sup> 27			
June 17					37	33					
June 23									40		
July 8			49	57	25	33		<sup>a</sup> 43	<sup>a</sup> 6		
July 13							39			47	
Aug. 2								62			
Aug. 5			57	53	<sup>a</sup> 39	<sup>a</sup> 47					
Aug. 17	71	86							48		
Aug. 30										66	
Sept. 15			48	54	38	44					
Sept. 19		79									
Sept. 22											52
Oct. 29 <sup>a</sup>	47	7	13	32	21	23	42	35	32	47	34
1927											
Apr. 30 <sup>a</sup>	26	17	0	3	0	0	10	2	3	14	18

<sup>a</sup> Weeds counted but no treatment.

It appears that little or nothing is gained by applying sodium chlorate before the plants are approximating the bloom stage. For example the treatment of plot 24, which was sprayed on June 2 when the plants were in full bloom and again on August 2, was more effective than the earlier treatment of plot 23, and almost as effective as the four applications on plots 21 and 22 which were first treated on May 14. Likewise the three applications to plots 6 and 7 of Table 1, beginning August 19, 1925, was but slightly more effective. The treatment of plot 25, which consisted of two applications, the first of which was made on June 23, was essentially as effective as that of plot 24.

When the first application was not made until after July 1, the treatment was much less effective than when treatments began in the bloom stage. This is illustrated by plots 11 and 26 as compared with plots 24 and 25. The reason perhaps is the severe drought, which, as previously mentioned, prevented normal growth. From a practical standpoint applications should not be delayed sufficiently to allow a seed crop to mature.

The results from plots 17 and 20 indicate that the weaker solutions are not effective enough for economical eradication. Thus two applications of the stronger solution as on plots 24 and 25 were as effective as the seven applications of the weaker solutions on plots 17 and 20.

A practical demonstration of the effectiveness of sodium chlorate in eradicating bindweed was made by the department of horticulture of

the Kansas State Agricultural College in 1926. A roadway 100 rods long through an orchard bordered by a heavy growth of bindweed was treated three times beginning at the period of full bloom. The weeds were completely eradicated.

#### EXPERIMENTS WITH SODIUM HYPOCHLORITE

The successful experiments with sodium chlorate suggested the possibility of using a cheaper material, which would react in a similar manner. An opportunity to conduct such experiments presented itself in the spring of 1926 and accordingly five additional plots were laid out. One of these was treated with "K. M. G. weed killer" and the others with 1, 2, 3, and 4 per cent solutions, respectively, of sodium hypochlorite. The results are presented in Table 3.

TABLE 3.—*Number of bindweed plants per quadrat in plots treated with sodium hypochlorite or with K. M. G. weed killer; April 30, 1926, to May 2, 1927*

Date of treatment	Number of bindweed plants per quadrat				
	Plot 19; sodium hypochlo- rite, 1 per cent	Plot 18; sodium hypochlo- rite, 2 per cent	Plot 13; sodium hypochlo- rite, 3 per cent	Plot 16; sodium hypochlo- rite, 4 per cent	Plot 15; K. M. G. weed killer
1926					
Apr. 30	30	39	53	80	70
May 11	48	64			
June 2	36	53	46	61	30
June 14	55	56	47	78	43
July 12	32	62	50	72	48
Aug. 8					52
Sept. 18	26	41	45	57	57
Oct. 19	33	56	46	58	54
1927					
Apr. 30 <sup>a</sup>	42	23	14	52	66

<sup>a</sup> No treatment.

The earlier applications of sodium hypochlorite were regarded as of little value because of the difficulty in preparing this material and the lack of uniformity in concentration. Later in the season it was prepared by using liquid chlorine. In this way it was possible to control the process and make a uniform product. The last two applications were made with material prepared in this way. The stronger solutions killed many of the bindweed, but none were as effective as the sodium chlorate. It is thought that still stronger solutions will be more effective, and future work will include such solutions. The effect on the appearance of the bindweed is very similar to that produced by sodium chlorate; that is, the plants die very slowly, assuming first a sickly yellow appearance followed by gradual dying of the leaves and finally by death. K. M. G. weed killer had no appreciable effect in reducing the number of bindweed.

#### EFFECT OF HERBICIDES ON THE SOIL

In view of the effectiveness of sodium chlorate in eradicating the bindweed it seemed desirable to determine whether it and other herbicides had any deleterious effect on the soil. Accordingly 11 of

the plots were sampled to a depth of 7 inches in May, 1926, and the nitrifying power of the soil determined. The results are presented in Table 4.

TABLE 4.—*Milligrams of nitrate per 100 gm. of soil in plots receiving different treatments*

Plot No.	Treatment	Milligrams of nitrate (NO <sub>3</sub> ) per 100 gm. of soil			
		Present when sampled	Formed during incubation		
			With water	With water and ammonium sulphate	With water and cottonseed meal
15	K. M. G. weed killer.....	0.88	8.78	29.48	52.24
16	Sodium hypochlorite, 4 per cent.....	1.95	3.72	4.48	15.15
1	Copper sulphate, 12.5 per cent.....	.62	7.05	59.04	70.21
2	Fallow.....	5.15	6.65	37.35	65.68
3	Zinc chloride, 12.5 per cent.....	3.24	7.94	32.17	83.94
4	Sodium arsenite, 10 per cent.....	7.46	11.96	86.98	59.21
5	Control.....	.44	21.35	84.56	105.81
6	Sodium chlorate, 25 per cent.....	24.96	13.61	23.36	69.48
17	Sodium chlorate, 6.25 per cent.....	7.08	11.81	16.53	28.33
18	Sodium hypochlorite, 2 per cent.....	4.53	10.38	13.18	37.97
21	Sodium chlorate, 12.5 per cent.....	4.72	7.60	11.02	15.21

It will be noted from these results that the soil from all plots contained nitrates when sampled. When these samples were incubated additional nitrates were formed, showing that bacterial action had not been seriously interfered with by the various treatments. There is some evidence of a retarding influence of the treatment in case of plots 16, 17, 18, and 21. These plots were treated with sodium chlorate and sodium hypochlorite a short time before the soil samples were taken. The effect is particularly noticeable where ammonium sulphate and cottonseed meal were added before incubation. That the retarding influence is temporary is indicated by the fact that it is not apparent in plot 6 which had received a heavy application of sodium chlorate the previous fall.

Plots 6 and 7 on which bindweed had been killed by sodium chlorate, were prepared and sown to wheat in September, 1926. This crop made a normal growth throughout the fall and came through the winter in excellent condition. So far as could be observed the sodium chlorate had no deleterious effect upon the growth of wheat.

#### SUMMARY AND CONCLUSIONS

Experiments to determine the relative effectiveness for the eradication of field bindweed of fallow, sodium chlorate, sodium arsenite, sodium hypochlorite, zinc chloride, copper sulphate, and K. M. G. weed killer were conducted. Various concentrations were used and observations were made as to the frequency and time of application. The roots were examined microscopically, drawings were made, and food reserves were studied. The results indicate that sodium chlorate is unusually promising for the eradication of field bindweed.

The optimum time for the application of the first spray is probably about the time the plants are in full bloom.

There seemed to be no permanent detrimental effect upon the soil from any of the chemicals used in these experiments.

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## SUPERNUMERARY CHROMOSOMES IN *ZEA MAYS*<sup>1</sup>

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### INTRODUCTION

*Zea mays* has been found to have a haploid chromosome number varying between 10 and 15 and corresponding variations in the somatic number.

Kuwada (13, 14, 15, 16),<sup>2</sup> the pioneer investigator of chromosomes in maize, abandoned in 1919 the possibility that 12-chromosomed sweet varieties of *Zea* represented a primitive type—a fact overlooked by the writer in previous articles.

The view that 10 is the primitive chromosome number in maize has been supported in recent articles by Longley (17, 18), Kiesselbach and Petersen (11), Fisk (9, 10), Reeves (22), and Randolph (21).

The writer investigated several old and well-established varieties of maize, all of which had uniformly 10 chromosomes, before finding some with supernumerary chromosomes. Plants with a somatic number of more than 20 chromosomes occur occasionally in the varieties Golden Bantam and Stowell's Evergreen and more frequently in Country Gentleman, Black Mexican, White Sheath, a strain of Pawnee flour corn, and in a highly inbred strain of White Dent (Crosby). The present paper reports on the character and behavior of extra chromosomes based on a three-year study of chromosomes in microspore mother cells in *Zea mays*.

### MATERIAL AND METHOD

The plants for this study of maize were grown at the Arlington Experiment Farm, Rosslyn, Va., and in the department's greenhouses in Washington, D. C. Many varieties and strains were examined, but the six above-named varieties or strains were the only ones in which plants with a haploid number of more than 10 were found.

The determination of chromosome numbers was confined to microspore mother cells. To make certain that haploid numbers found in mother-cell material were constant for individual plants, preparations were made from the major tassel and from the tassels of suckers of the same plant. Except for occasional abnormal-appearing cells, all parts of a plant gave the same chromosome number. Consequently, after trying this test on many plants of several varieties without finding serious discrepancies, the writer felt assured that by determining the chromosome number from microspore mother cells of a

<sup>1</sup> Received for publication June 13, 1927; issued December, 1927.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," page 783.

plant one could satisfactorily ascertain the chromosome number of individual plants.

The method used in the laboratory of making cytological preparations of mother-cell material has passed through an evolutionary stage during the last three years. Very early it was found that the microspore mother-cell material of *Zea* lends itself very satisfactorily to the use of iron-aceto-carmines as a killing and staining method. This method, however, necessitated the use of fresh material and so restricted study to the short period when corn was in flower.

To overcome this difficulty and to lengthen the period of study, material was killed in 70 per cent alcohol, and it was found that with this poorly balanced killing fluid the material was sufficiently well preserved to be useful in making iron-aceto-carmines preparations even after the material had been stored in alcohol for months.

The next step was to substitute acetic-absolute (1 to 3) killing fluid for 70 per cent alcohol. The specimens are left in this fluid 20 minutes and then washed with absolute alcohol. Thompson (29) has also described this method of preserving material for future study when stained with iron-acetocarmine. It was found that excellent preparations could be made from material killed in this manner. The mother cells were not as pliable as those stained from fresh material, but by careful staining they could be kept very much longer without deterioration. In fact, the deterioration of slides was generally due to a drying out of the staining liquid. This results in a concentration of the stain to the point of crystallization, giving particles of carmine that may be confusing.

A modification of Mann's (20) method for making permanent mounts has recently been tried and found completely satisfactory. Slides are cleaned, a light layer of fixative applied, and then anther material previously killed in acetic-absolute solution is macerated in a drop of water on the slide. The microspore mother cells are teased out, and with the edge of a cover glass the anther wall fragments are removed, leaving the mother cells floating in water. The slide is now dried in the same manner as a slide covered with paraffin sections. The mother cells seem to withstand the treatment quite as satisfactorily as those embedded in paraffin. After the slide has dried for an hour or more it can be stained, dehydrated, and mounted in balsam in the usual manner.

Carmine-stained preparations have some advantages over material stained with haematoxylin. The chromosomes are semitransparent when stained with carmine, and the strands making up an individual can be traced better than when stained with haematoxylin. Small grains found in the nuclei of some preparations, which resemble chromatin fragments when stained with haematoxylin, fail to take a carmine stain, and so this confusion is overcome by using the latter stain.

Slides are studied by the aid of a one-sixteenth objective and  $\times 8$ ,  $\times 15$ , and  $\times 18$  oculars. The drawings used in the figures were made with a camera lucida, magnification 1,500.

#### CHROMOSOME CHARACTERISTICS

A detailed study of the chromosomes of *Zea mays* at diakinesis made it apparent that chromosome individuality was distinguishable. Fisk (10), in her recent article, has opened the way by describing some

of the types which bivalent chromosomes assume. These shapes are illustrated in Plate XI, Figure 34, of her article.

The bivalent chromosomes at diakinesis in their partially contracted condition are sufficiently constant in shape to allow a comparison of individual chromosomes in the same nucleus as well as in different nuclei. Just prior to the fading out of the nucleolus, eight of the chromosomes are simple rings or rings with the two ends overlapping. Six of these generally appear as rings, although at times they are not completely closed. The chromosomes A, B, E, F, G, and H of Fisk's (10) drawing seem to correspond to these six rings. Two are rings with a pronounced overlapping of the two ends and possibly correspond to chromosomes C and I of her diagram.

Of the 10 chromosomes the 2 remaining ones are more characteristic. One is large and often appears as a figure 8, which Fisk (10) describes as "larger and slightly more twisted." The tenth is a small chromosome and has no easily described shape.

Figure 1, A-F, shows a few illustrations in which all 10 chromosomes are at a stage when their characteristic shapes can be best recognized. B shows clearly the two ring-shaped chromosomes with overlapping ends and the figure 8 chromosome, but two of the six ring-shaped chromosomes are very open and are hardly recognizable.

Figure 2, A and B, has been arranged to show the constancy of the chromosome shapes in four different microspore mother cells of the same plant as well as in two related plants. The chromosomes have been arranged according to the scheme apparent in Fisk's drawing (10, *pl. XI, fig. 34*). The minor variations apparent in the attempted comparison are either partially or wholly due to the usual difficulties experienced in finding cells in the same stage of development, chromosomes in the same stage of contraction, and chromosomes in a favorable plane for showing their characteristic shapes.

Figure 3, A-F, shows figures from mother cells with more than 10 chromosomes. The more outstanding chromosome shapes can be identified. Each set shows the eight open or closed ring-shaped chromosomes and the figure-8 chromosome. The supernumerary chromosomes are distinguishable from any of these two classes, but frequently resemble so closely the small tenth chromosome of the normal set that identification becomes uncertain. In some prophase figures the two units of an extra chromosome are very loosely held together, and occasionally they are separate and appear as two univalent chromosomes. A and B show a pair of univalent chromosomes. B and F are drawings of chromosomes from plants with odd somatic number which consequently show an unpaired univalent present. Univalents generally can be recognized by their small size and compact globular shape.

Figure 2, C, is a continuation of the chromosome comparison of A and B. Each of the four sets illustrated shows extra chromosomes at the end of the series. The first has five supernumerary chromosomes, all approximately the same size and resembling most nearly the smallest of the 10 normal chromosomes. Their shapes are in no way characteristic and for this reason are not confused with the ring-shaped and the figure-8 chromosomes.

This study of chromosome shapes in *Zea mays* has been confined to the first reduction division of the microspore mother cell. Chromosomes at the metaphase stage (fig. 4) assume shapes which give some indications of the point of spindle fiber attachment. Eight

chromosomes have central or subterminal points of fiber attachments. These eight seem to correspond with the eight ring-shaped chromosomes. Those rings with overlapping ends seem to correspond in number with those that have subterminal but not central points of



FIG. 1.—Diakinesis in the microspore mother cells of *Zea mays*, showing characteristic shapes assumed by the 10 bivalent chromosomes: A, Golden Bantam sweet corn; B, Black Mexican sweet corn; C, Red X Crosby; D, Wichita flint; E, Hybrid Ah-645; F, Country Gentleman sweet corn

fiber attachments, while the more nearly perfect ring-shaped chromosomes have central fiber attachments. The two remaining chromosomes have approximately terminal points of fiber attachment. One, a large chromosome, corresponds to the figure-8 chromosome of

diakinesis, and the other, the smallest of all chromosomes, corresponds to the tenth small chromosome already referred to.

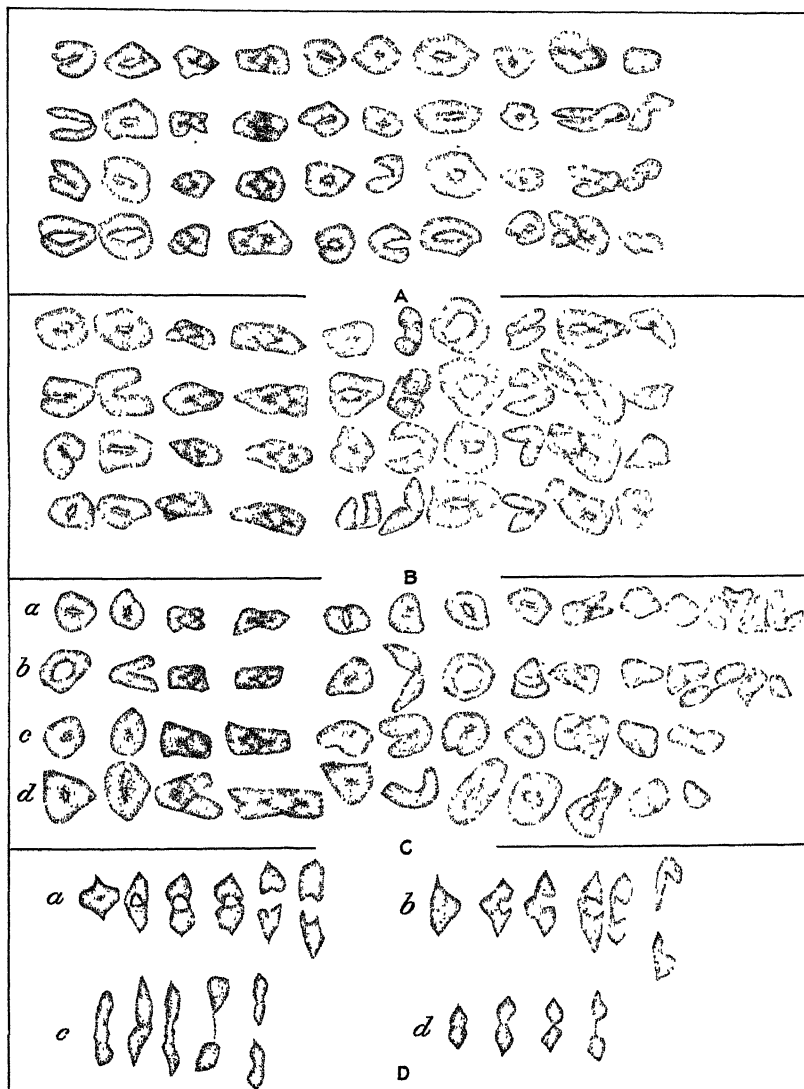


FIG. 2.—Bivalent chromosomes of maize: A, Four chromosome sets from hybrid Ah-645-1748, arranged to show the similarity of chromosome shapes in four microspore mother cells of the same plant; B, four chromosome sets from hybrid Ah-645-1751; C, a, a chromosome set from a plant of Black Mexican sweet corn with 15 bivalent chromosomes; b, a similar set from a sister plant with a univalent and 13 bivalent chromosomes; c, a set from a third sister plant with 11 bivalent chromosomes; d, a chromosome set from Country Gentleman sweet corn with a univalent and 10 bivalent chromosomes; D, chromosomes from heterotypic metaphase plates of microspore mother cells, showing the region of fiber attachment: a, Stages in the division of chromosomes with central fiber attachment; b, stages in the division of chromosomes with subterminal but no central fiber attachment; c, a larger chromosome with terminal fiber attachment; d, a smaller chromosome with terminal fiber attachment

Figure 2, D, illustrates the differences apparent in chromosomes during their separation at the heterotypic division. Large chromosomes with central spindle fiber attachments split first at the middle.

The final points of separation are the two extremities (D, a). When the fiber is attached subterminally but not at the center, the split starts

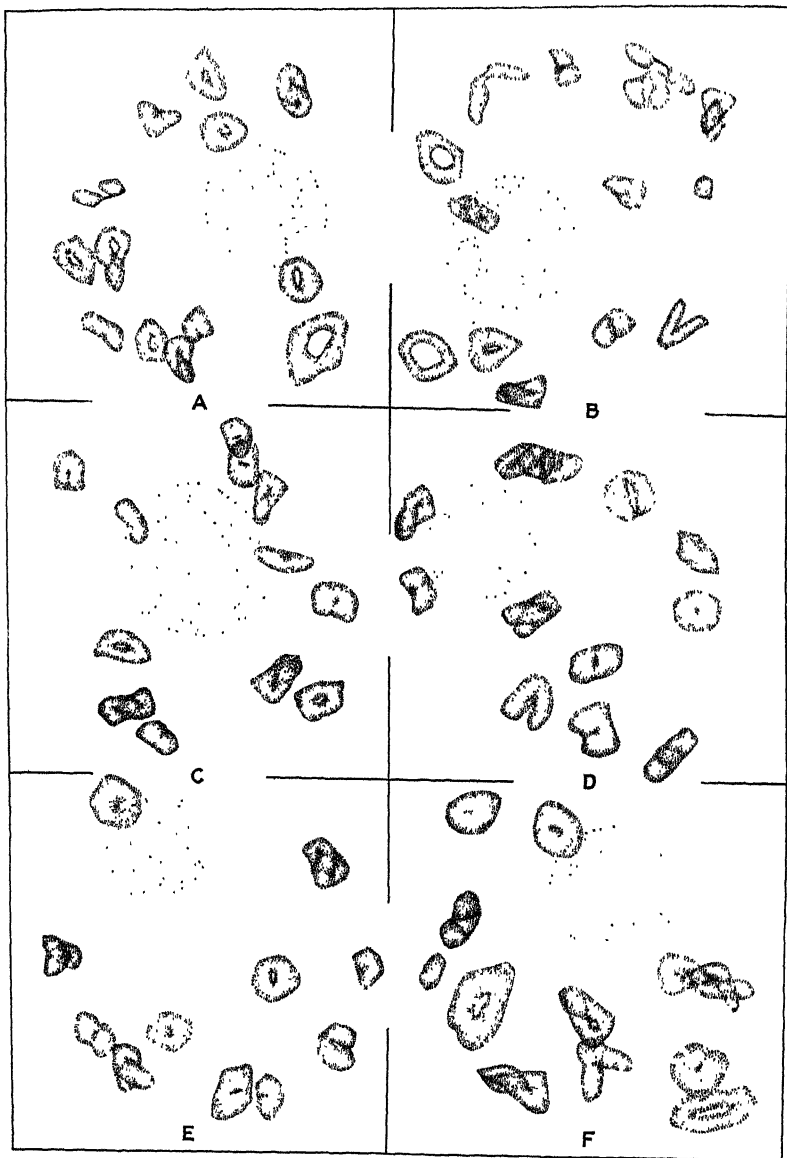


FIG. 3.—Diakinesis in the microspore mother cells of *Zea mays*, showing the characteristic shape of both the normal and the supernumerary chromosomes: A, Black Mexican sweet corn, showing 13 bivalent chromosomes; B, Black Mexican sweet corn, showing a univalent and 13 bivalent chromosomes; C, Black Mexican sweet corn, X Dwarf ( $F_7$ ), showing 12 bivalent chromosomes; D, Black Mexican sweet corn, showing 12 bivalent chromosomes; E, Black Mexican sweet corn, showing 11 bivalent chromosomes; F, Golden Bantam sweet corn, showing a univalent and 10 bivalent chromosomes

toward one end, and at the final stage the two units are held together at one extremity only (D, b). The two chromosomes with terminal fiber attachments are best distinguished by their size and rodlike

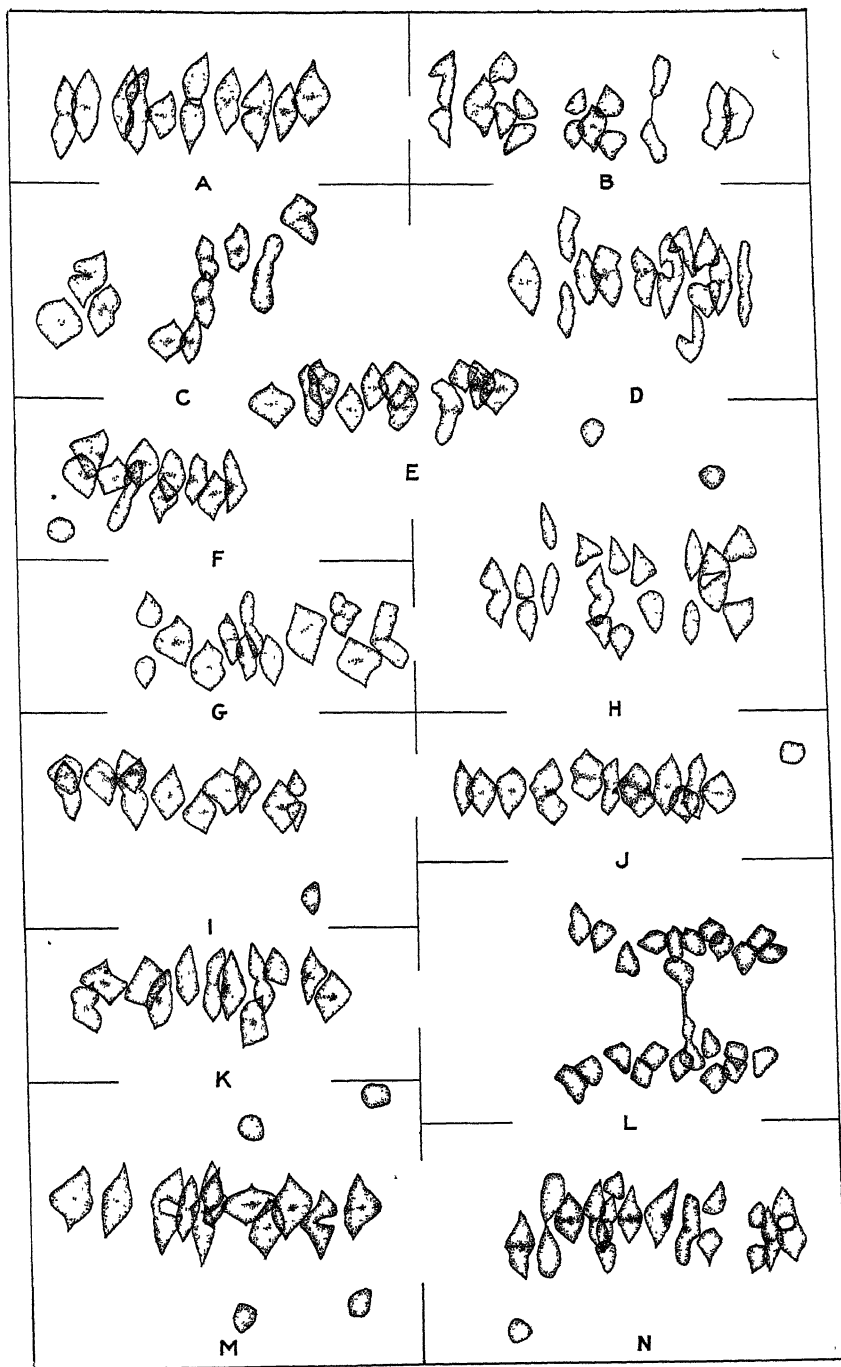


FIG. 4.—Heterotypic metaphases from microspore mother cells of *Zea*, showing the shape and behavior of chromosomes at this phase: A and C, Country Gentleman sweet corn with 10 bivalent chromosomes; B and D, Black Mexican sweet corn with 10 bivalent chromosomes; E and G, Country Gentleman sweet corn with a univalent and 10 bivalent chromosomes; F, Country Gentleman sweet corn with 11 bivalent chromosomes; H, Hybrid Ph-285 with 2 unpaired and 10 paired chromosomes; I, Country Gentleman sweet corn with a univalent and 11 bivalent chromosomes; J, Red×Crosby (F<sub>2</sub>) with a univalent and 11 bivalent chromosomes; K, Red×Crosby with 12 bivalent chromosomes; L, Black Mexican sweet corn (anaphase) with 12 divided chromosomes; M, Black Mexican sweet corn with 4 unpaired and 11 paired chromosomes; N, Black Mexican sweet corn with a univalent and 13 bivalent chromosomes

shape. The large one is shown in D, *c*, the smaller in D, *d*. Fisk (10) finds one small chromosome that occasionally divides early, a behavior that has been noticed in the tenth small chromosome and for supernumerary chromosomes. Consequently, supernumerary chromosomes are classified with the small chromosome of the normal 10, due to similarity in size, shape, and mode of division at the heterotypic metaphase.

### BEHAVIOR OF SUPERNUMERARY CHROMOSOMES

Before attempting to describe the behavior of supernumerary chromosomes during meiosis, it seems in order to describe the normal behavior of chromosomes in plants with the basic number 20. Fisk (10, *Table 1*), reports that in progenies usually characterized by 20 somatic chromosomes some cases in which more or less than 10 chromosomes possibly are present at diakinesis were found, and only one case of nondisjunction in the homotypic division which gave rise to pollen cells with 9 and 11 chromosomes. Only infrequently has the writer found such irregularities in the number and behavior of chromosomes at diakinesis in microspore mother cells of plants with 20 chromosomes. It is likely that through such irregularities in chromosome distribution plants with more than 20 chromosomes have arisen.

A study of the offspring from plants with 20 chromosomes grown at Arlington in 1926 failed to give any plants with more than the normal number. Some of these were descendants of plants carrying several supernumerary chromosomes, but evidently the grandparents did not carry factors that affected the distribution of the 10 basic chromosomes, since none of the F<sub>2</sub> plants had more than 20 chromosomes. A similar study of 21 progenies in 1924 gave one exception—six plants were grown from a Black Mexican parent supposed to have 20 chromosomes. Five of these plants had 22 chromosomes and one had 21. Unfortunately, all the cytological material from the parent was used in making the determination, thus preventing a confirmation of this chromosome count. Consequently, there was no means of determining whether an error in observation had been made or whether these plants with extra chromosomes were the offspring of a plant with 20 chromosomes. The chromosome number of the six plants indicates the former.

TABLE 1.—*Distribution by chromosome number of 97 plants, both parents having 20 chromosomes, some of the grandparents having more than 20 chromosomes*

Progeny designation	Designation of grandparents		Designation of parents		Chromosome number of grandparents		Chromosome number of parents		Chromosome classes of progeny	
	Female	Male	Female	Male	Female	Male	Female	Male	20	20+
P-2-----	(a)	(a)	532	532			20	20	29	0
P-83-1-----	C-1	C-1	89-B <sub>4</sub>	89-B <sub>3</sub>	20	20	20	20	14	0
P-83-2-----	C-1	C-1	89-B <sub>3</sub>	89-B <sub>2</sub>	20	20	20	20	7	0
P-83-3-----	D-5	D-5	94-A <sub>18</sub>	94-A <sub>20</sub>	21	21	20	20	25	0
P-83-4-----	G-4	B-4	97-A <sub>6</sub>	97-A <sub>5</sub>	24?	21	20	20	22	0

\* Commercial seed.

The figures given in Table 1, taken from material collected in 1925, indicate that the 20 constantly present chromosomes of maize are regular in their behavior at meiosis. Exceptions similar to that found the previous year must be of rare occurrence. Consequently, in this study of the inheritance of a supernumerary chromosome, irregularities in the distribution of the 20 chromosomes of the normal set are regarded as insignificant.

The data contained in Table 2 are in marked contrast with those in Table 1. When the parent plant has 22 instead of 20 chromosomes,  $F_1$  plants with from 20 to 24 chromosomes are found instead of 22, the number which would be found if the eleventh chromosome behaved as regularly during the reduction division as the 10 normal chromosomes.

TABLE 2.—*Distribution by chromosome number of 73  $F_1$  plants, both female and male parents having 22 chromosomes*

Progeny designation	Designation of parent plants		Chromosome number of parents		Chromosome classes of $F_1$ offspring				
	Female	Male	Female	Male	20	21	22	23	24
P-2-1.....	8035	8035	11	11	-----	3	1	1	-----
P-2-2.....	8067	8067	11	11	-----	-----	3	2	5
P-2-3.....	8226	8226	11	11	1	1	-----	-----	-----
P-2-4.....	8284	8284	11	11	5	2	1	1	-----
P-83-1.....	G-4	G-4	11	11	-----	2	2	-----	-----
Ab-848-1.....	556B	556B	11	11	-----	10	5	25	-----
Ab-848-2.....	280-B <sub>18</sub>	280-B <sub>23</sub>	11	11	-----	1	1	1	-----
Total.....	-----	-----	-----	-----	6	19	13	30	5

In order to analyze the apparent irregularities in the behavior of supernumerary chromosomes, the simplest case was chosen. Plants with 21 chromosomes were crossed reciprocally with plants with 20 chromosomes and with each other, by which process three groups of progenies were obtained—those in which the female parent carried one extra chromosome, those in which the male parent carried the extra chromosome, and finally those in which both parents carried an extra chromosome. The chromosome number was determined in a large number of these  $F_1$  plants. Tables 3, 4, and 5 show the number of plants obtained in the various chromosome classes of these three related groups.

In the five progenies given in Table 3 only the male parent had an extra chromosome. The presence of a few plants with 22 chromosomes in four of the five progenies studied shows that the distribution of the extra chromosome at the time of pollen formation in the male parent was irregular. Regular division of a supernumerary chromosome in either the heterotypic or the homotypic division in the microspore mother cell of the male parent would give in these crosses equal numbers of 20 and 21 chromosomed plants. This is clearly not the case, for in four of the five progenies studied the number of 20-chromosomed plants was much in excess of those with 21 chromosomes, and there is present a third class of plants with 22 chromosomes. It seems unlikely that a differential death rate has been effective in eliminating pollen grains with extra chromo-

somes, since in progeny P-83-2 the 11-chromosomed pollen, effective in bringing about fertilization, outnumbers the 10-chromosomed grains, and in progeny P-2-2 there is one 12-chromosomed pollen grain to three 10-chromosomed grains. The ratio in the latter is that expected when the extra chromosome nondisjunctions in the homotypic division.

TABLE 3.—*Distribution by chromosome number of 112 F<sub>1</sub> plants, the female parent having 20 and the male parent 21 chromosomes*

Progeny designation	Designation of parent plants		Chromosome number of parents		Chromosome classes of F <sub>1</sub> offspring			Percentage of nondisjunction
	Female	Male	Female	Male	20	21	22	
P-2-1-----	C <sub>8</sub>	B <sub>1</sub>	20	21	10	2	2	69.5
P-2-2-----	64-B <sub>3</sub>	64-B <sub>4</sub>	20	21	24	0	8	100.0
P-4-1-----	508-3	508-4	20	21	23	11	4	42.1
P-83-1-----	94-B <sub>3</sub>	94-B <sub>3</sub>	20	21	13	0	1	100.0
P-83-2-----	95-A <sub>7</sub>	95-A <sub>17</sub>	20	21	6	9	0	0

<sup>a</sup> Calculated from the ratio of the two observed classes.

Table 4 shows a similar behavior for this extra chromosome when present in the megaspore mother cell. There are many more 20-chromosomed plants than 21-chromosomed plants in all progenies studied, and again there are a few 22-chromosomed plants. These facts indicate an irregular behavior of the extra chromosome at meiosis of the megaspore mother cell of 21-chromosomed plants similar to that noticed in the microspore mother cell. In the production of an embryo-sac mother cell, however, three of the four cells of the linear tetrad die. All four of the progeny ratios of Table 4 show a deficiency in 12-chromosomed cells, indicating that both a differential death rate, which eliminates some of the cells with extra chromosomes, and nondisjunction have been effective in distorting the observed ratios between the three chromosome groups.

TABLE 4.—*Distribution by chromosome number of 170 F<sub>1</sub> plants, the female parent having 21 chromosomes and the male parent 20 chromosomes*

Progeny designation	Designation of parent plants		Chromosome number of parents		Chromosome classes of F <sub>1</sub> offspring			Percentage of nondisjunction
	Female	Male	Female	Male	20	21	22	
P-2-1-----	64-B <sub>4</sub>	64-B <sub>3</sub>	21	20	19	11	0	26.70
P-83-1-----	93-B <sub>13</sub>	93-B <sub>15</sub>	21	20	27	12	1	36.00
P-83-2-----	94-B <sub>7</sub>	94-B <sub>1</sub>	21	20	31	21	2	24.40
P-83-3-----	95-A <sub>5</sub>	95-A <sub>16</sub>	21	20	32	14	0	39.1
Total-----					109	58	3	26.8

<sup>a</sup> Calculated from the ratio of the two observed classes.

Table 5 gives different classes of plants resulting from a union of pollen and eggs, both produced from plants with 21 chromosomes. A regular behavior of the supernumerary chromosomes would give plants with the chromosome numbers 20, 21, and 22, in the proportion 1 : 2 : 1. A glance at the observed populations shows that not only

is the ratio of 20, 21, and 22 chromosomed plants much distorted but also that a fourth class with 23 chromosomes has appeared.

These additional data, therefore, confirm the assumption that an extra chromosome is erratic in its behavior at meiosis of both the megaspore and the microspore mother cell.

TABLE 5.—*Distribution by chromosome number of 274 F<sub>1</sub> plants, both female and male parents having 21 chromosomes*

Progeny designation	Designation of parent plants		Chromosome number of parents		Chromosome classes of F <sub>1</sub> offspring				Percentage of nondisjunction	
	Female	Male	Female	Male	20	21	22	23	Female <sup>a</sup>	Male
P-2-1.....	50-B <sub>8</sub>	50-B <sub>4</sub>	21	21	14	5	3	3	26.8	100
P-2-2.....	69-B <sub>10</sub>	69-B <sub>4</sub>	21	21	26	14	19	6	26.8	100
P-33-1.....	95-A <sub>17</sub>	95-A <sub>18</sub>	21	21	12	19	13	0	26.8	0
P-33-2.....	96-A <sub>12</sub>	96-A <sub>12</sub>	21	21	20	20	4	2	26.8	35
P-33-3.....	97-A <sub>11</sub>	97-A <sub>11</sub>	21	21	16	11	10	2	26.8	65
P-33-4.....	97-A <sub>17</sub>	97-A <sub>9</sub>	21	21	17	22	6	1	26.8	10

<sup>a</sup> Value taken from Table 4.

Cytological observations of microspore mother cells with 21/2 chromosomes have shown that the extra univalent does not divide in the first of the reduction divisions. This observation is substantiated by the study of F<sub>1</sub> progenies one or both parents of which had 21 chromosomes. If this extra chromosome divided and the halves went to opposite poles in the first reduction division, there would be produced pollen and eggs with 10 and 11 chromosomes only. This can not be the case since in the F<sub>1</sub> progenies are found plants that must have originated from pollen and eggs with 12 chromosomes. Frequently in microspore mother-cell preparations with an extra chromosome, one of the chromosomes is found nondisjoining in the homotypic division. It is this behavior, combined with the failure of a supernumerary chromosome to divide in the heterotypic division, that produces some pollen and eggs with 12 chromosomes. There is thus provided a reasonable explanation of the irregular chromosome behavior that brings about the aberrant ratios found in this hybrid offspring.

Figure 5, A and B, is a schematic drawing which shows the sequence of events in the production of cells with extra chromosomes in the linear and in the pollen tetrads.

The figures given in Tables 3 and 4 show that nondisjunction has significantly modified the expected ratios of the progenies studied. A more critical consideration of these aberrant ratios made it apparent that the amount of nondisjunction for individual progenies may be very definitely estimated. For example, progeny P-4-1, Table 3, has 23 plants with 20 chromosomes, 11 with 21 chromosomes, and 4 with 22 chromosomes. The excess of the first over the second class is 12 plants. This excess added to the 4 plants of the third class, gives 16 plants out of a total of 38 that, within the limits of random sampling, must have originated through nondisjunction. There was in this progeny, therefore, 42.1 per cent of nondisjunction.

When possible, the percentages of nondisjunction were calculated by Fisher's formula (8, p. 25) for determining the maximum likelihood. The percentages of nondisjunction thus calculated are given in the last column of each table.

Table 3 records the behavior of an extra chromosome in microspore mother cells. The percentages vary from 0 to 100, while those for the megaspore mother cell, recorded in Table 4, vary only from 20 to 36. These percentages suggest that the extra chromosome when present in the less-protected microspore mother cell is more erratic in its behavior than when present in the well-protected megaspore mother cell. The individual behavior of the five progenies listed in Table 3 are so different that they can not rightly be classed

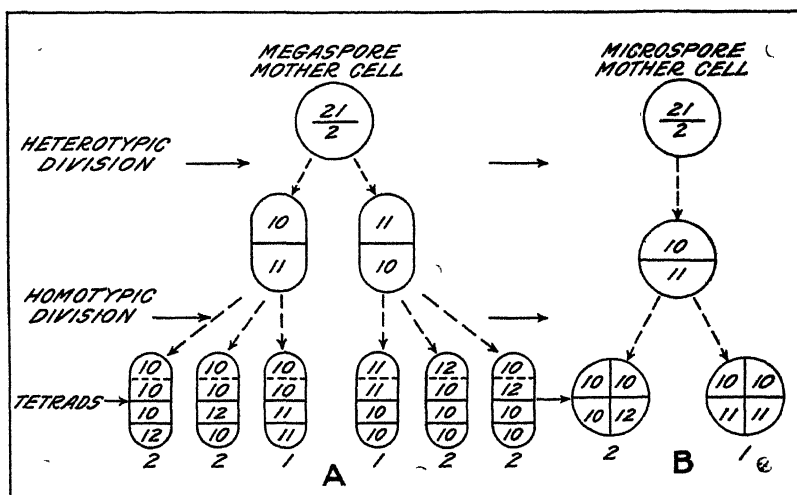


FIG. 5.—Diagram showing distribution at meiosis of  $21\frac{1}{2}$  chromosomes to the four cells of a tetrad: A, Megaspore mother cells. The first division gives two cells, one with 10 and the other with 11 chromosomes. The second division gives six types of tetrads, two (see A-1) in which the division of the eleventh chromosome is normal and four (see A-2) in which there is nondisjunction of the two halves of the eleventh chromosome; B, microspore mother cells. The first division gives two cells, one with 10 and the other with 11 chromosomes. The second division gives two types of tetrads, one (see B-1) in which the division of the eleventh chromosome is normal and one (see B-2) in which there is nondisjunction of the two halves of the eleventh chromosome.

together, while the four progenies listed in Table 4 are not significantly different, which permits their combination into one group and the calculation of the total percentage of nondisjunction for the combined group. For the megaspore mother-cell material studied this percentage is 26.8, while the percentage for the microspore mother-cell material is variable, ranging from 0 to 100 per cent.

Progenies P-2-1, listed in Table 4, and P-2-2, listed in Table 3, may be used for comparing the behavior of this extra chromosome in the megaspore and microspore mother cells of the same plant. The percentage of nondisjunction for the former is 26.62, while that for the latter is 100. Compared by Fisher's (8, p. 84)  $X^2$  test of Independence, these two progenies give an  $X^2$  of 10.106+, indicating that there exists a significant difference in the reciprocal cross.

## DISCUSSION

Variations in the form and size of chromosomes have frequently been referred to and pictured by cytological investigators. Sharp (23, p. 173) discusses individual chromosome characteristics, and his simile comparing a chromosome set to a series of unlike wheels in a watch seems very apt. Just such a set of 10 chromosomes and apparent differences in anatomical characteristics of sister chromosomes are to be found in maize.

Carothers (5) in *Circotettix*, Taylor (24, 25, 26, 27, 28) in *Fritillaria*, *Abstroemeria*, and other genera, and Ferguson (7) in *Aloinae* have recently investigated anatomical chromosome characteristics. One outstanding character found constant in chromosomes is the region of fiber attachment and the associated constriction. Taylor (28) considers the heterotypic division unfavorable for detecting constrictions. However, the region of the fiber attachment shows distinctly at the heterotypic metaphase of microspore mother cells of *Zea mays*. This character is constant for each chromosome and serves as a useful mark for chromosome identification. In all diploid forms of maize there are constantly two chromosomes with approximately terminal, two with subterminal, and six with central regions of fiber attachment.

Shapes of chromosomes, such as rods, V's, and J's, for somatic chromosomes and double V's or rings, double J's, X's and 8's for bivalent chromosomes, are described by Belling (2, 3) for hyacinths. These characteristic shapes are not peculiar to hyacinths, but are observed by cytologists in innumerable plant forms. When seen at diakinesis in a favorable plane the bivalent chromosomes of *Zea mays* can be classified as six rings or double V's, two loops with overlapping ends or double J's, and a figure 8.

Size of chromosomes is also a much-used characteristic for identifying individual chromosomes and even whole chromosome sets. Babcock and Lesley (1) find similarity of chromosome size is a useful criterion of relationship of *Crepis* species. Outstanding size differences are apparent in *Zea* chromosomes. The six rings have appreciable size differences, the figure-8 chromosome is large, and one chromosome of the normal set is most readily identified by its small size.

The determination of definite chromosome characteristics for each of the 10 chromosomes making up the set of all diploid forms of maize should make it easier to identify and trace the behavior of extra chromosomes in aneuploid forms. Supernumerary chromosomes in maize have terminal points of fiber attachment, are small, and in general resemble closely the tenth small chromosome of the normal set.

In order to determine whether supernumerary chromosomes are new bodies or simple duplications of chromosomes of the normal set, recourse may be had to genetic analysis. Such an analysis is now in progress.

The present study is concerned with the inheritance of a single extra chromosome. Carother's (5) article on the distribution of chromosomes in *Circotettix* has demonstrated that the laws of heredity apply to the segregation of chromosomes making up a normal set. Belling (2) has shown that in a triploid hyacinth the

chromosomes in excess of the diploid number are distributed by chance. Watkins (30, 31) and Kihara (12) have studied the behavior of unpaired chromosomes in *Triticum* hybrids, and have found that although they are distributed at meiosis by the law of chance, there is a differential death rate eliminating pollen with extra chromosomes.

These observations of *Zea mays* indicate that one extra chromosome in a megaspore or a microspore mother cell is affected by some factor that modifies the chance distribution. The different possibilities have been tested, and the conclusion reached that nondisjunction of the extra chromosome in the homotypic division causes the production of some pollen and eggs with 12 chromosomes. It has been found that the percentage of nondisjunction for a particular progeny can be determined approximately from the ratios between 10, 11, and 12 chromosomed gametes. These percentages show that nondisjunction of an extra chromosome in the second division of the microspore mother cells in the five progenies studied is variable, ranging from 0 to 100 per cent, while in the four progenies of megaspore mother cells nondisjunction was observed to occur in approximately 26 per cent of the cells carrying an extra chromosome.

Outstanding examples of plants in which pollen grains with a certain chromosome number are more effective in bringing about fertilization than other grains with a lower or a higher number are *Triticum*, already referred to; *Oenothera lata*, in which Lutz (19) believes that only male gametes with seven chromosomes function; the Globe mutant of *Datura*, found by Buchholz and Blakeslee (4) to transmit the extra chromosome in about 3 per cent of the pollen grains; and *Nicotiana tabacum*, in which Clausen and Goodspeed (6) observed a similar elimination of pollen carrying an extra chromosome. In maize there seems to be no indication that male gametes with extra chromosomes are less effective in bringing about fertilization than those having the normal set of 10. In the selection of an embryo-sac mother cell from the linear tetrad, however, there are definite indications of a differential death rate which increases the number of 10-chromosomed female gametes at the expense of those carrying 12 chromosomes.

These data on the distribution of more than one supernumerary chromosome are too incomplete to be included in the present article.

#### SUMMARY

Ten has been found to be the haploid chromosome number present in most maize varieties and forms. These 10 normal chromosomes, when viewed in the heterotypic division, have individual characteristics such as size, shape, and position of fiber attachment. This chromosome set is composed of six double V's, two double J's, a figure 8, and a small chromosome with no easily describable shape.

The behavior of the normal chromosome set during the reduction phases is, with rare exceptions, regular.

A few maize forms have been found in which, frequently or infrequently, plants appear with supernumerary chromosomes.

Extra chromosomes resemble in size and shape the smallest of the normal chromosome set.

The distribution of a supernumerary chromosome to the four daughter cells has been studied and found to be erratic.

It goes undivided to one or the other of the poles in the first reduction division, and nondisjunction occurs in varying percentages in the second division.

In microspore mother cells nondisjunction varies from 0 to 100 per cent in the five progenies studied.

In megaspore mother cells nondisjunction is a more stable factor and is found to be approximately 26 per cent in the four progenies studied.

Pollen viability seems to be in no way associated with chromosome number. On the other hand, a differential death rate seems to eliminate part of the embryo-sac mother cells with two supernumerary chromosomes.

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# DEVELOPMENT OF THREE MIDSEASON VARIETIES OF CABBAGE RESISTANT TO YELLOWS (*FUSARIUM CONGLUTINANS* WOLL.)<sup>1</sup>

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## INTRODUCTION

The nature and increasing destructiveness of cabbage yellows (*Fusarium conglutinans* Woll.) throughout the central belt of States from coast to coast have already been amply discussed in previous reports (2, 3, 4).<sup>4</sup> It is now 15 years or more since the improvement of cabbage varieties with respect to their resistance to this disease was begun by Jones and his associates. The first efforts with the late-storage type resulted in the development of Wisconsin Hollander (2), while selections from two standard sauerkraut varieties have led to the introduction of Wisconsin All Seasons and Wisconsin Brunswick (3). These three varieties are now in common use where the yellows disease prevails and where the needs are met by these types. In each case they are late-maturing varieties eminently suited for the production of late storage cabbage on the one hand and a late sauerkraut crop on the other.

In many sections where yellows occurs, the early and midseason crops equal or exceed the late crop. It is therefore natural that with the increasing importance of the disease a keen demand has arisen for resistant strains maturing earlier than those in use and conforming in type to the somewhat varied demands. Accordingly, as already noted (4), selection work was begun some years ago from a number of the earlier varieties, there being now included in the list All Head Early, Glory of Enkhuizen, Copenhagen Market, Jersey Wakefield, and Charleston Wakefield.

Progress with the first three varieties named has reached the point where certain of the selections not only possess high degrees of resistance to the yellows organism, but also have reasonable uniformity in other respects such as time of maturity, shape and quality of head, color, and productiveness. It has therefore been found expedient to multiply the seed of these lines and to introduce them to the trade channels, where it is expected that they will fill a place as midseason varieties in yellows-infested regions. These new strains resemble in their main features the varieties from which they were selected. They possess the added character of resistance and some variations as to type, and these differences seem to warrant varietal distinction. The names of the new varieties which will be

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<sup>4</sup> Reference is made by number (italic) to "Literature cited," p. 808.

used in the present discussion and which are recommended for general usage are listed below along with the names of the parent varieties from which they were derived.

<i>Yellows-resistant variety</i>	<i>Parent variety</i>
All Head Select.	All Head Early.
Globe.	Glory of Enkhuizen.
Marion Market.	Copenhagen Market (midseason strain.)

The purpose of this paper is to describe the methods used in the development of the new varieties and to record the salient details of the experimental work which have yielded the present results.

#### PRINCIPLES OF CABBAGE BREEDING INVOLVED

In cabbage the blossoms are open-pollinated and as a rule are cross-fertilized by insects. The writers' experience with artificial self-pollination of many cabbage seed plants has shown that in the main they are only slightly self-fertile, but sufficiently so to produce enough seed for pure-line selections. In cases where certain strains have been repeatedly selfed for three generations, some reduction in size of plants has been noted. Thus, continued pure-line selection may have some disadvantage where the stimulation of heterosis seems to be essential to productiveness. As a result the usual method employed for the improvement or maintenance of a standard variety of cabbage is first to select a number of individuals possessing certain desirable characters. These are reset the following season in an isolated plantation where cross-pollination between individuals may occur. By planting the seed of each plant separately the third season, individuals from the best of these may be selected and the process repeated. Thus there is attained a gradual approach toward homozygosity for certain characters, while vigor is maintained by allowing the plants from selected rows to cross-pollinate.

This method of selection has been followed with unusual success in the work of Jones and his associates (2, 3) in the development of the three *Fusarium*-resistant varieties already mentioned, Wisconsin Hollander, Wisconsin All Seasons, and Wisconsin Brunswick. Although in the commercial varieties from which these strains were selected only an occasional plant showed resistance to the disease, the progenies of such individuals were highly resistant in the second or third generation. Strains completely free from susceptible plants were never attained, however, by this method.

More recent work (8) indicates that resistance is dominant over susceptibility. Moreover, a study of the  $F_2$  generation of crosses between highly resistant and highly susceptible parents shows that segregation occurs at a ratio very close to 3 resistant to 1 susceptible, and therefore indicates that resistance and susceptibility are allelomorphic unit characters (8). One would therefore expect to encounter difficulty in completely eliminating the recessive character of susceptibility by means of the head-to-row selection from open-pollinated plants.

In the development of the three resistant late varieties already mentioned (2, 3) some marked deviations in type have resulted. This is most striking in the Wisconsin Hollander, which was selected

from two individuals of Ferry's Danish Ballhead variety. The latter is a late-maturing storage type which produces a spherical, compact head with comparatively few outer leaves and a short stem. The Wisconsin Hollander, by way of contrast, matures some two weeks later than Ferry's Danish Ballhead and produces a taller, ranker plant with a much larger head which is elliptical rather than spherical in vertical cross section. The Wisconsin All Seasons matures somewhat later than the standard All Seasons and in spite of repeated head-to-row selection still varies considerably as to shape of head, leaf color, and uniformity in maturing. One of the chief reasons for these deviations probably lies in the fact that the disease reduced the plants available for the original selections to a very small group within which the opportunity to select for conformity to the standard type was decidedly limited. This change was so marked in the initial step with Wisconsin Hollander that it has seemingly been impossible so far to bring the resistant strain to the original type of the standard commercial variety through head-to-row selection from individuals allowed to open-pollinate.

The deviations in type which obtain in Wisconsin Hollander, Wisconsin All Seasons, and Wisconsin Brunswick are fortunately not so great as to impair their value in meeting certain commercial needs. The problem before the writers has been to produce supplementary resistant varieties earlier in maturity and of various types. Selections from the earlier standard varieties, according to the method just outlined, showed a tendency for the strains to become later in maturity as they became more resistant. It therefore seemed evident that self-pollination and controlled cross-pollination of selected individuals offered possibilities for improvement which might not be attained by the other method. It should be possible to eliminate the undesirable recessive character of susceptibility by this means, while improvement in type by getting rid of certain undesirable characters might be expected. Should it be necessary to self-pollinate for many successive generations in order to reach the objective in mind, reduction in size and vigor of plants might occur. On the other hand, this obstacle might be overcome, if necessary, by carrying two or more distinct lines toward the same objective and then crossing two such lines when they had reached the desired degree of homozygosity, in order to restore vigor through heterosis.

#### METHODS OF EXPERIMENTATION

Cabbage under most conditions is a biennial. The simple and practicable method in the past was to make initial selections for resistance from a commercial field of the desired variety in which yellows had been very severe. These seed heads were placed in storage and set out the following spring and allowed to become cross-pollinated. Seed from each plant was saved separately and planted the following season on thoroughly sick soil in adjacent rows. The best plants from the most resistant rows were again saved and the process repeated until a highly resistant type was obtained. During the progress of the work now reported, certain revisions of this method have been adopted.

## SEED PROPAGATION

The seed heads, instead of being held over winter in storage, are placed in the greenhouse for seed propagation during the winter months. This method offers three distinct advantages. It advances the rate of progress by allowing the progenies of one year's selection to be tested the following year. It precludes the necessity of prolonged winter storage of seed heads, which is especially difficult with the early, poorly keeping varieties. It facilitates greatly the practice of controlled pollination.

As the plants in the field approach maturity sufficiently to be judged for type, they are pulled and either planted in pots at once or more often held for some weeks in a cool cellar or in artificial cold



FIG. 1.—Cabbage seed plants in the greenhouse at Madison, Wis., April, 1926

storage. There is little advantage in potting the plants immediately, inasmuch as they require several weeks of dormancy before they go into flower and seed production. The potted plants are kept at comparatively low temperatures ( $10^{\circ}$  to  $15^{\circ}$  C.) in order that the root system may become well established before the rapid growth of the top takes place. Plants selected during August and September are planted during October and are placed out of doors where they are allowed to remain until there is danger of injury from freezing. They are then removed to the greenhouse and kept at  $10^{\circ}$  to  $15^{\circ}$  C. until near blossoming time, when the temperature is raised to about  $20^{\circ}$ . By this means it is ordinarily possible to bring plants into blossom at Madison, Wis., in February. (Fig. 1.)

The manipulation of pollination is carried out in the usual manner. A portion of the seed plant, usually the central stem, is bagged and the pistils are artificially self-pollinated by working a camel's-hair brush over the open blossoms on alternate days. Such branches as are selected for crossing are bagged before blossoming begins, and

the buds are emasculated before the anthers become mature. Inasmuch as the indeterminate raceme grows rapidly at this stage, it is necessary to emasculate daily to avoid selfing. The pistils are receptive for several days, so that daily emasculation with pollination every second or third day ordinarily results in a satisfactory set of seed. As soon as the seeds have passed the "milk stage" the plants are cut and hung up in a warm, airy house to hasten maturity. By this means a majority of plants are ready to thresh by the last of May. Such seed may be sown at once, and plants are ready to set into the field during the last week of June or early July. Under Wisconsin conditions this program usually allows a thorough test for resistance and type during the current summer. Greenhouse propagation of cabbage seed has also been used with success by Melhus and his associates (5) at the Iowa Agricultural Experiment Station.

#### FIELD TESTS FOR RESISTANCE AND TYPE

In conducting the field tests, certain lines of procedure have developed during a period of several years which may well be described in some detail. The influence of soil temperature upon the incidence of cabbage yellows has been studied by Gilman (1), by Tisdale (7), and by Tims (6). In experiments where the soil was held at constant temperatures over a certain gradient it was found that the disease did not occur at 15° C., that it progressed slowly at 17°, and that it developed with greatest rapidity at 26° to 29°. The Wisconsin Hollander and Wisconsin All Seasons showed less resistance under these experimental conditions as the temperature was increased, and at the optimum for disease development there was nearly a complete breakdown in resistance. Of course, the entire root system is seldom if ever exposed to this extreme condition in nature. There were some differences in the severity of the disease at various soil moistures (7). In the soil which had a water-holding capacity of 31 per cent, the disease developed most rapidly and destructively at 15 per cent moisture, which was below the point where good normal growth of the plant was sustained; it was nearly as severe at 19 per cent moisture, where the best growth of the host occurred. At the latter soil-moisture content 100 per cent of the seedlings showed yellows, and 93 per cent had succumbed to the disease at the end of 28 days. In still higher moistures the disease was less destructive. At 23 per cent moisture an average of 89 per cent showed yellows and 64 per cent died, while at 26 per cent moisture 97 per cent were diseased and 68 per cent were killed. In the light of these facts, soil temperature and soil moisture may be expected to influence the development of the disease in the field as well as the expression of the resistant character.

Ordinarily under southeastern Wisconsin conditions the soil temperature is too low during late spring to permit development of yellows in the cabbage seed bed, while the most rapid development of the disease occurs usually in July during the three or four weeks following transplanting. On the other hand, in an occasional season the temperature during the middle or latter part of June is high enough to favor infection. In such an instance, were the seed bed made on infested soil, certain of the susceptible individuals would be eliminated before transplanting and more plants would succumb

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in the moderately resistant and susceptible progenies than in the highly resistant ones. This would introduce an error into the comparison of those progenies in the field. The seed bed is therefore made on yellows-free soil and the plants transferred to infested soil for the resistance tests. Through a period of years it has been found that the most uniform and critical tests for resistance in the locality mentioned are obtained by sowing the seed from May 10 to May 30 and transplanting from June 25 to July 15.

The same plot of soil located in eastern Kenosha County, Wis., is used each year in making the field tests. By repeated cropping with cabbage this soil has become as thoroughly and uniformly infested with the yellows organism as is readily possible to attain under natural field conditions. For purposes of comparison a commercial (susceptible) variety and one of the resistant varieties now in general use, such as Wisconsin All Seasons, are planted at frequent intervals throughout the plot. The customary procedure in notation of yellows has been to determine the number of diseased plants in each progeny at intervals of 7 to 12 days after the first appearance of the disease. A gradual increase occurs in the number of diseased individuals until about the middle of August, the rate depending partly upon climatic conditions. During this period a gradation in the severity of the disease among individuals of a given progeny is usually noticeable. A number of the diseased plants are often killed within a few days after the appearance of the first symptoms, some persist for a few weeks, a few even produce small heads at the end of the season, while still others may show only slight symptoms on one or two lower leaves during the warmest part of the season. The last-mentioned type of plant eventually loses the affected leaves, and thereby any outward sign of the disease disappears, and with the return of cooler weather subsequent growth is normal. Whether this variation is a true gradation in resistance due to differences in hereditary factors, or whether certain environmental factors are responsible in whole or in part, is still an unsettled question. It is certain that soil temperature, soil moisture, and stage of growth of the host at the time of infection are important factors which influence the expression of resistance.

Variation from season to season and variation in time of planting within a single season have been found to influence the expression of resistance of a given progeny, and undoubtedly these differences are traceable to the varied effects of these environmental factors. It has therefore become a common practice, where the seed supply permits, to test the important progenies through two or three seasons. All plants which show even slight symptoms of yellows are labeled with small bamboo stakes as soon as the disease appears, so that if they recover later in the season they are avoided in making further selections. This custom of permanently marking all diseased plants was first adopted in 1924. (Fig. 2.)

Beginning with 1924, the plants affected by yellows were divided into four more or less arbitrarily established classes, namely, (1) those killed by the disease, (2) those so severely injured as to be unable to form heads, (3) those only slightly affected, which may form heads although they clearly show slight inhibitive effects of the organism, and (4) those in which the slight symptoms disappear during the course of the season and which head quite normally. This classification gives

a clearer picture of the damage caused by the disease in a particular strain than would be apparent in the mere record of the total percentage of plants affected. Thus, in a given strain showing 25 per cent of the plants affected, all or most of the cases might fall in the "recovery" class, whereas in another strain showing the same percentage of plants affected the majority might fall in the "dead" class. For purposes of comparison in this paper, the classification is simplified somewhat. In the field results of 1924 and subsequent



FIG. 2.—A portion of the Kenosha trial plot on yellows-sick soil. Note bamboo stakes used to mark each plant showing yellows. The two rows in the center contain highly resistant families, while at either side are very susceptible families

seasons, the plants falling in the "dead" and "severe" classes are combined in one category, and the "slight" and "recovery" classes are combined in a second group.

#### DESCRIPTION OF PARENT STOCKS USED

Cabbage varieties are commonly divided with respect to the length of their period of development into three groups—early, second early or mid season, and late. As previously noted, the need for yellows-resistant late varieties has been met for the present, and the work in progress has to be primarily with the mid-season and early sorts. In this report attention is given to the mid-season varieties. The market and sauerkraut demands within this group are not sufficiently uniform to justify concentrated attention upon selection within a single mid-season variety. In sauerkraut manufacture the preference is strongest for the flathead type, while in many cases the market demands are decidedly in favor of the roundhead varieties.

It seemed advisable, therefore, to make selections from varieties representing both types. For the flat type the All Head Early variety was chosen because of its popularity among sauerkraut packers and in certain cabbage-shipping sections. For the roundhead type Glory of Enkhuizen and Copenhagen Market were used. These last two are grown widely for mid-season shipping and for sauerkraut manufacture. Copenhagen Market properly belongs in the early group, but, as will be pointed out later, certain strains sold under this name really belong to the mid-season class. It is from one of these mid-season strains that the selections from Copenhagen Market herein described were made. A brief description of each of these parent varieties is given.

#### GLORY OF ENKHUIZEN

The variety known as Glory of Enkhuizen was introduced into America in the late nineties by Sluis & Groot, a seed firm of Enkhuizen, Netherlands. It produces a prominent spherical head which at maturity has a light-yellowish cast, and in most strains now in use there is sufficient "bloom" to give a bluish green tinge to the outer foliage. The stem is comparatively short, and at maturity the outer leaves are not very numerous, resulting in the characteristic prominence of the head. This variety has become very popular in recent years as a shipping cabbage following the early crop, such as early Copenhagen Market, Jersey Wakefield, and Charleston Wakefield, and preceding the late varieties such as Danish Ballhead. It is also used widely in sauerkraut manufacturing centers where it commonly shares in popularity with All Head Early and the mid-season strains of Copenhagen Market for the early pack of sauerkraut.

#### COPENHAGEN MARKET

Certain strains sold under the name of Copenhagen Market are close to Glory of Enkhuizen in type and maturity, and sometimes the two varieties are used interchangeably for market and for sauerkraut manufacture. Copenhagen Market was introduced into America in 1912 by Hjalmar Hartman & Co., of Copenhagen, Denmark. The original introduction was an early-maturing roundheaded cabbage with yellowish green foliage. Like the Glory of Enkhuizen, it produces a prominent head and comparatively few outer leaves at maturity. The original type, however, matures at least two weeks earlier than Glory of Enkhuizen and ordinarily produces a smaller head. Since the time of its introduction much deviation from the original type has appeared in many of the strains offered for sale. This has probably been brought about by laxity in selection, by mixture, and by substitution. In any case, at the present time two general types of Copenhagen Market are recognized. Certain of the strains now listed by seedsmen as "Extra Early Copenhagen," "Superselect Copenhagen," "Reselected Copenhagen," or with other qualifying terms conform closely to the original. Golden Acre, a recent introduction, seems to differ little from the original Copenhagen Market. Other strains produce larger heads, are more variable as a rule, and mature anywhere from a few days to two weeks later than the original strain; but they are still sold as Copenhagen Market. Such strains approach the characters of Glory of Enkhuizen and

really belong in the mid-season group. In this connection it may be well to cite the opinion of Work (9):

Those who plant Copenhagen cabbage should take account of the fact that there are two distinct types of this variety. The one is early as Wakefield, is small, [and] matures very uniformly \* \* \*. Golden Acre is practically the same as this strain, being, if anything, smaller and earlier. The second type is larger, later, and an excellent strain, but is sure to disappoint the man who works for early market. This is a type that is best suited for mid-season market and for the kraut factory.

In the present investigation with this variety original selections were made at Marion, Va., from stock labeled Copenhagen Market. It was evident from the beginning, however, that it was one of the later strains of the variety, and it was continued because of its intrinsic value as a mid-season type for shipping and local market as well as for sauerkraut. Thus the resistant strain Marion Market, though selected from Copenhagen Market, does not possess the early-maturity qualities of certain strains of the latter, and this fact should be clearly recognized in its use. Selection has been started from very early strains of Copenhagen Market, but these are being carried as distinct lines and will be reported upon later. The resistant strain Iacope, introduced recently by Melhus, Erwin, and Van Haltern (5), was also derived from Copenhagen Market.

#### ALL HEAD EARLY

The original selection of All Head Early was made in 1888 by W. Atlee Burpee, from a field of Henderson's Succession being grown in eastern Long Island by J. M. Lupton. It was introduced to the trade in 1891. It is one of the earliest of flathead types of cabbage and for that reason has become a popular variety for early sauerkraut. In the lots of this variety which have been studied considerable variability has been noted, and it does not appear to be a well-fixed variety. As a whole it may be considered as having a short stem, flat head with comparatively few outer leaves at maturity, petioles short, and foliage intermediate in color between the yellowish green of Copenhagen Market and the bluish green of Glory of Enkhuizen. Certain undesirable variations from this type will be discussed in connection with the detailed account of selections made from this variety.

#### DEVELOPMENT OF THE RESISTANT VARIETY GLOBE FROM GLORY OF ENKHUIZEN

##### FIRST-GENERATION SELECTIONS

Initial selections were made in 1921 from resistant individuals which remained healthy in a planting of very uniform commercial stock of Glory of Enkhuizen on the trial grounds in Kenosha County, Wis. This soil was thoroughly infested with the yellows fungus, and only a small percentage of the original plants survived. From these, several heads were selected which conformed reasonably well with the average type for the variety. They were stored over winter and planted the following spring in an isolated spot where cross-pollination was allowed to take place. Head-to-row tests were made in 1923 from the six plants producing seed. The results, recorded in Table 1, show that all progenies had considerable resistance.

Retrials in 1924 and 1925 indicate that the last two seasons offered conditions for a more severe test than did 1923. These first selections all conformed reasonably well to the standard type. Two progenies, however (35-1 and 2) showed a tendency of the heads to flatten slightly, and they were therefore discarded. From the four remaining lots about 100 heads were saved for greenhouse seed propagation in the winter of 1923-24.

TABLE 1.—*Behavior of first, second, and third generation progenies of Globe selected from Glory of Enkhuizen variety of cabbage on yellows-infested soil*

Generation	Parent strain	Progeny number *	1923 trials		1924 trials			1925 trials			1926 trials		
			Total number of plants	Yellows (per cent)	Total number of plants	Yellows, dead or severe (per cent)	Yellows, slight or recovered (per cent)	Total number of plants	Yellows, dead or severe (per cent)	Yellows, slight or recovered (per cent)	Total number of plants	Yellows, dead or severe (per cent)	Yellows, slight or recovered (per cent)
First	Commercial Glory of Enkhuizen.	35-1	246	16	---	---	---	---	---	---	---	---	---
		35-2	254	18	219	15	5	65	23	6	---	---	---
		35-3	255	20	40	28	3	49	22	6	---	---	---
		35-4	254	14	---	---	---	---	---	---	---	---	---
		35-5	257	21	46	15	0	26	31	0	---	---	---
		35-6	513	15	---	---	---	---	---	---	---	---	---
Second	35-3 to 6	35-11s	---	---	24	13	0	26	4	8	---	---	---
		35-11×12	---	---	105	1	0	75	1	1	46	0	0
		35-12s	---	---	69	0	0	52	0	2	---	---	---
		35-12×11	---	---	25	0	0	219	0	0	---	---	---
		35-12×13	---	---	---	---	---	51	0	0	---	---	---
		CG-7×35-11	---	---	62	13	0	---	---	---	---	---	---
		CG-7×35-12	---	---	50	0	0	---	---	---	---	---	---
		CG-7×35-13	---	---	67	42	15	---	---	---	---	---	---
		35-12×11	---	---	---	---	---	---	---	---	18	22	0
		35-22s	---	---	---	---	---	25	0	0	17	0	0
Third	35-12s	35-22×21	---	---	---	---	---	---	---	---	---	---	---
		35-43s	---	---	---	---	---	---	---	---	8	0	0
		35-43×59	---	---	---	---	---	---	---	---	13	0	0
		35-51s	---	---	---	---	---	---	---	---	20	25	0
		35-58s	---	---	---	---	---	---	---	---	21	0	0
		35-59s	---	---	---	---	---	---	---	---	19	0	0
		35-67s	---	---	---	---	---	---	---	---	17	0	0
		Commercial	251	73	48	60	10	214	53	30	240	61	27
Wisconsin All Seasons.		---	258	1	55	0	2	50	0	4	23	0	0
		---	---	---	---	---	---	---	---	---	---	---	---

\* The suffix s indicates that the progeny is the result of self-pollination of a given plant. When a progeny is the result of a cross between two plants, e. g., 11×12, the number of the pistillate parent is given first. Progenies 35-1 to 35-6 are from plants 1 to 6 which were allowed to cross-pollinate.

<sup>b</sup> Further selections were made at the end of the season

<sup>c</sup> CG-7 was a plant from the commercial variety Glory of Enkhuizen, the selfed progeny of which showed practically 100 per cent yellows on sick soil, indicating that it was homozygous for susceptibility.

#### SECOND-GENERATION SELECTIONS

The mortality<sup>5</sup> of seed plants in the greenhouse was very heavy, and only two of the second-generation selections produced seed (35-11 and 12), while a third (35-13) produced enough pollen to use in certain crosses. In addition to selfing each plant and crossing within the group, crosses were made with a plant of a susceptible

<sup>5</sup> Decay of the stem in the region of and below the head is common in the greenhouse. It occurs after the mother seed heads are planted and either during the dormant period or after elongation of the seed stem has started. No single organism is responsible for this injury. Bacteria and fungi, including *Phoma lingam* and *Rhizoctonia* spp., are commonly associated with the decay.

strain. Tests of these progenies were made in 1924 and 1925, and the results are included in Table 1.

The progeny from the selfing of plant 35-11 showed some susceptibility to yellows, while progeny 35-12s was highly resistant.

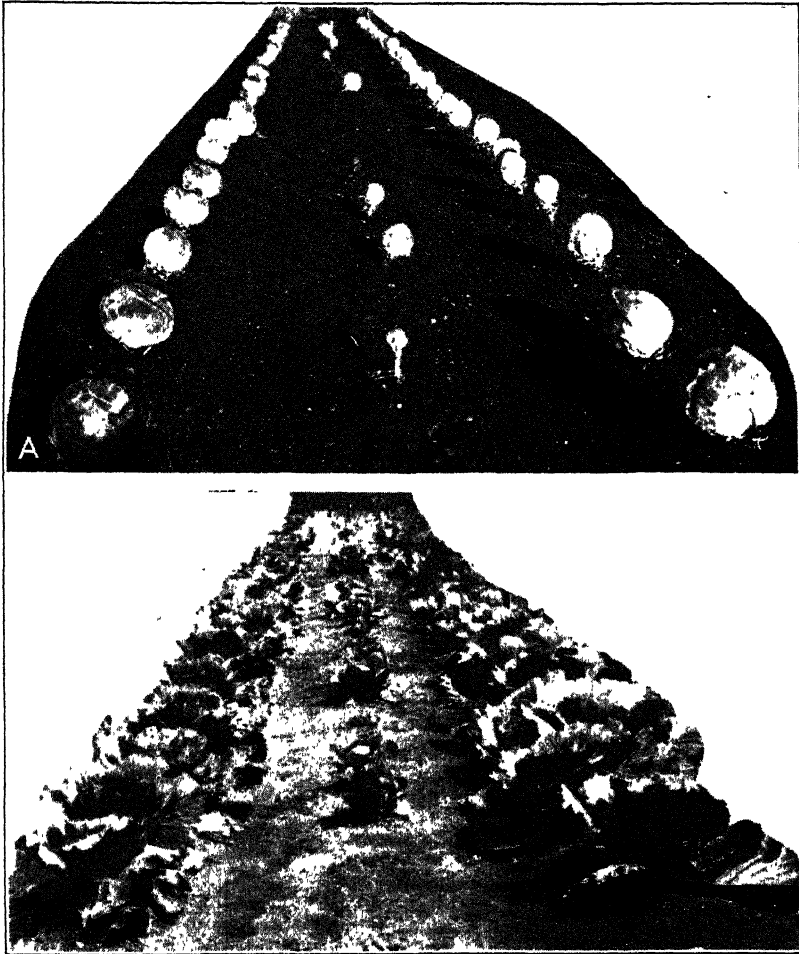


FIG. 3.—Progeny tests of midseason cabbage strains on Kenosha trial plot in 1925. Bamboo markers were removed before the photographs were taken: A, Center row is commercial Copenhagen Market, which showed 96 per cent of plants yellowed. At the right is Globe progeny 35-12x11 (see Table 1), selected from Glory of Enkhuizen completely free from disease. At the left are hybrids from a cross between 35-12 which was homozygous for resistance and a Copenhagen Market plant which was heterozygous for resistance. Note that resistance is completely dominant. B, Center row is commercial Golden Acre, of which 83 per cent of the plants were diseased. At the left is Marion Market progeny 30-13x15B (selected from Copenhagen Market), which showed one slight case of yellows out of 47 plants. At the right are All Head Select progenies, which were highly resistant.

Hybrid plants from crosses in which plant 35-12 entered into the combination also proved to be highly resistant. In Figure 3, A, progeny 35-12x11 is shown in comparison with commercial Copenhagen Market and with the hybrids from a cross between a partially resistant plant and plant 35-12. Other experiments to be reported

later have shown that whenever a plant which is homozygous for resistance is crossed with a heterozygous one or one homozygous for susceptibility the  $F_1$  hybrid plants are resistant. It is to be noted here that when plant 35-12 was crossed with a susceptible plant the  $F_1$  hybrid plants were all resistant. This indicates that plant 35-12 was homozygous for resistance. The  $F_1$  hybrid from crosses between plants 11 and 13 and the susceptible parent showed marked degrees of susceptibility, indicating that they were still heterozygous for resistance. The selfed progeny from plant 12 (35-12s) was very uniform and satisfactory in type, and this was therefore used as a basis for further selections. Plants from this lot were saved in 1924 and 1925, and several third-generation progenies were thus obtained. In addition a number of plants from progeny 35-12 $\times$ 11 were saved and later cross-pollinated, the seed being grouped into one lot, 35-26-B.

#### THIRD-GENERATION SELECTIONS

Inasmuch as progeny 35-12 $\times$ 11 was the result of a cross between a homozygous and a heterozygous parent, a certain amount of reversion would be expected in the next generation. This proved to be the case when lot 35-26-B was tested and found to have 22 per cent yellows in the "dead or severe" class. (Table 1.) The progenies derived from 35-12s all proved to be 100 per cent resistant with the exception of 35-51s, which again segregated at the ratio of 3 resistant to 1 susceptible. This should not have been the case if plant 35-12 were truly homozygous. An explanation of this variation is offered by the possibility that it was the result of contamination in the pollination of plant 35-12. Such chance contamination is not entirely eliminated and was particularly likely to occur in 1924-25 greenhouse propagation, when all the selfed branches were not bagged during the entire blossoming period. The supposition is supported in this case by the facts that the segregation was exactly in the 3 to 1 ratio and this particular progeny showed marked deviation in type from 35-12s while the sister progenies continued to resemble the mother progeny quite uniformly. It is likely that with the exception of 35-51s the third-generation progenies are homozygous for resistance under field conditions.

#### TYPE OF THIRD-GENERATION PROGENIES

The third-generation progenies show a marked degree of uniformity in type. Typical heads are shown in Figure 4, A, B. The color is very close to that of the average Glory of Enkhuizen, while time of maturity and shape and texture of head all seem to be reasonably similar in the two strains. The prominence of the head at the time of maturity is not as pronounced in the selected progenies as is often noted in standard Glory of Enkhuizen. This is due largely to the fact that the new strain has a few more leaves in the outer foliage, which does not constitute a serious objection. With the exception of progeny 35-51s, all third-generation progenies resemble very closely the characters of 35-12s and 35-12 $\times$ 11. It is evident that pure-line selection for two successive generations has, in addition to fixing resistance, served to fix certain characters which go to make up type. By this means much of the variability in cabbage varieties

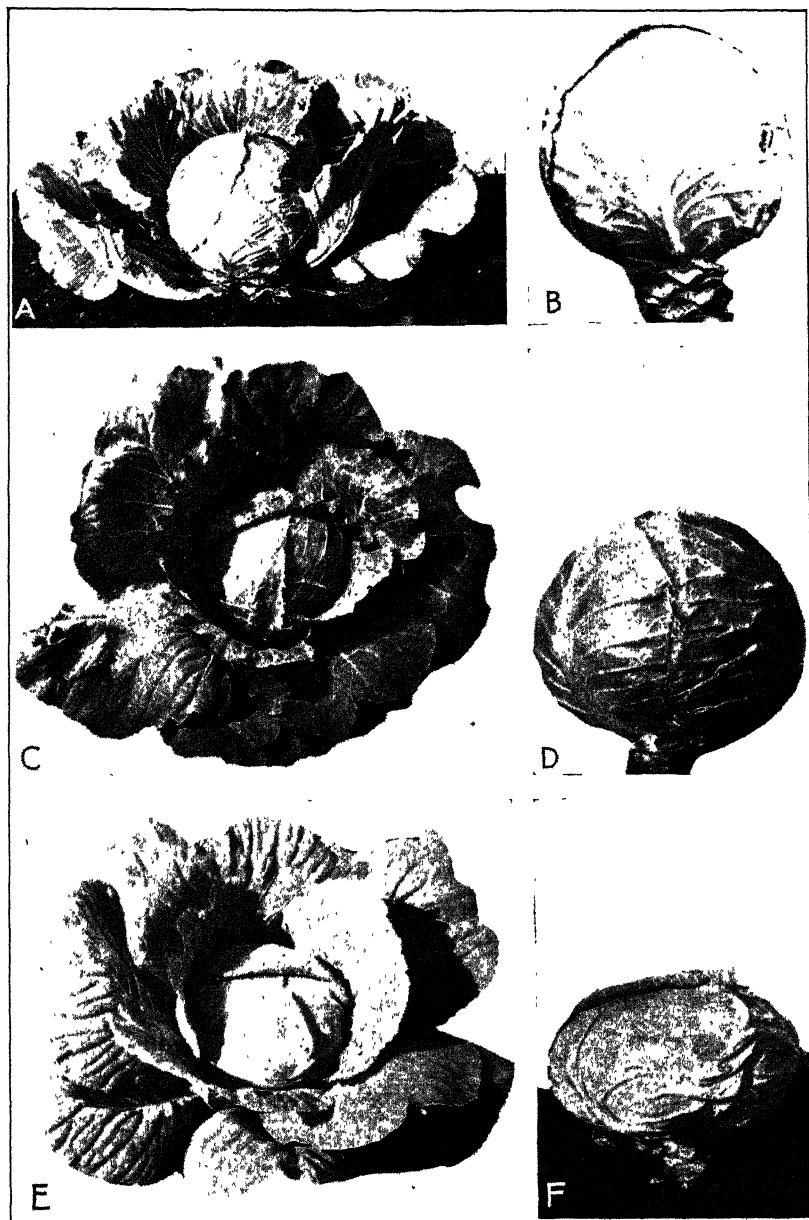


FIG. 4.—Typical plants from the yellows-resistant midseason varieties: A, B, Globe, a round-head type selected from Glory of Enkhuizen; C, D, Marion Market, a roundhead type selected from Copenhagen Market; E, F, All Head Select, a flathead type selected from All Head Early. Compare with Figure 5

may undoubtedly be eliminated. There is little evidence that the third-generation progenies are seriously reduced in vigor as a result of selfing for two successive generations.

#### MULTIPLICATION OF GLOBE

It is evident that the selections within this variety (to which the name Globe has been given) have reached a point where little may be expected from further pure lining. The present plan, therefore, is to multiply the third-generation progenies (except 35-51s). The plants from these progenies will be allowed to cross-pollinate in order to provide a greater quantity of seed and to insure against reduction in vigor. The seed thus procured may serve as stock seed for multiplication under proper safeguards in one or another cabbage-seed growing region.

Because of the urgent demand for resistant strains of this type a number of heads from strains 35-3 to 6 were selected for type in 1923, and seed was produced from them in 1924. A trial of this stock seed in the Kenosha plots in 1925 showed only 12 per cent of the plants severely diseased, while 80 per cent remained healthy, and the type, though not equal to the best progenies tested in 1926, was quite as satisfactory as commercial Glory of Enkhuizen. The remainder of this stock was sent to the Puget Sound region in the spring of 1925 by the National Kraut Packers' Association, and a crop of seed was obtained in 1926. Thus this advance lot of seed will be available at once for preliminary use on a commercial scale until the multiplied seed from the third-generation progenies becomes available.

#### DEVELOPMENT OF THE RESISTANT VARIETY MARION MARKET FROM COPENHAGEN MARKET

Initial selections were made in 1921 from a commercial planting of Copenhagen Market in a demonstration plot conducted near Marion, Va., under the direction of F. D. Fromme, of the Virginia Agricultural Experiment Station. The original stock was one of the later maturing strains of Copenhagen Market, having on an average a bluish green rather than a yellowish green foliage, although variation in this respect was evident from the beginning. Selection has now passed through three generations. The results of the trials on the Kenosha grounds are given in Table 2.

#### FIRST-GENERATION SELECTIONS

The original heads selected at Marion in 1921 were held over winter in storage and planted together in an isolated spot in 1922, where they were allowed to become cross-pollinated. Head-to-row progeny tests were made in 1923 from the six plants maturing seed (30-1, 2, 3, 4, 5, 6). All six progenies showed high resistance, the percentage of yellows varying from 11 to 18. Retrials of certain of the progenies in 1924 and 1925 showed higher percentages of disease, indicating that yellows was not generally as severe in 1923 as in the two following seasons. In the 1923 trials these progenies showed a fairly satisfactory approximation to the type of the late strains of Copenhagen Market. They were decidedly later than the early strains of the variety and in fact were scarcely earlier than the

first-generation progenies of the Globe selections from Glory of Enkhuizen tested on the same trial grounds in the same year. There was some variation in color, but in the main the plants were more bluish green than those of the standard Copenhagen Market. Two of the progenies (30-22-4 and 5) averaged somewhat later in maturity than the rest and showed some tendency to produce flattened rather than spherical heads and were therefore discarded. From the remaining four progenies about 100 of the best plants were saved for greenhouse seed production during the winter of 1923-24.

TABLE 2.—Behavior of first, second, and third generation progenies of Marion Market selection from Copenhagen Market variety of cabbage on yellows-infested soil

Generation	Parent strain	Progeny number <sup>a</sup>	1923 trials		1924 trials			1925 trials			1926 trials		
			Total number of plants	Yellows (per cent)	Total number of plants	Yellows, dead or severe (per cent)	Yellows, slight or recovered (per cent)	Total number of plants	Yellows, dead or severe (per cent)	Yellows, slight or recovered (per cent)	Total number of plants	Yellows, dead or severe (per cent)	Yellows, slight or recovered (per cent)
First	Commercial Copenhagen Market.	30-1	250	14	22	36	5						
		30-2	249	16	68	19	0	97	25	0			
		30-3	253	10									
		30-4	248	18									
		30-5	254	14									
		30-6	133	14									
Second	30-1, 2, 3, 4	30-12s			25	0	0						
		30-12×14B			62	0	0	50	0	0			
		30-13s						50	0	0			
		30-13×15B						47	0	4			
		30-14s			34	12	0	50	22	0			
		30-14×12B			29	17	0	212	25	3			
		30-14×17B						79	29	3			
		30-15s						50	18	8			
		30-15×13B						50	24	4			
		30-16s						50	0	0			
		30-16×18B						25	0	0			
		30-17s						75	24	0			
		30-17×14B						34	24	0			
		30-12s									84	0	0
		30-30s						26	0	0			
		30-31s						53	4	2			
		30-32s						50	15	2			
		30-33s						22	32	6			
		30-35s						25	24	4			
Third	30-13s	30-36s									51	0	0
		30-37s									49	0	0
		30-38s									114	25	0
		30-38×37									36	0	0
		30-40s									29	0	0
		30-41s									133	26	0
		30-40×41B									139	0	2
		30-41×40B									150	25	2
		30-53s									48	0	0
			251	73	48	60	19	214	53	30	240	61	27
Commercial Wisconsin, all seasons			258	1	55	0	2	50	0	4	23	0	0

<sup>a</sup> The suffix s indicates that the progeny is the result of self-pollination of a given plant. When a progeny is the result of a cross between two plants, e. g., 38×37, the number of the pistillate parent is given first. In some cases the blossom of the pistillate parent was not emasculated, but a brush was worked back and forth between blossoms of paired plants, and such progenies are therefore a mixture of plants resulting from selfing of the pistillate parent and from crossing with another staminate parent. Such a progeny is designated by the suffix B, e. g., 13×15B. Progenies 30-1 to 30-6 are from plants 1 to 6 which were allowed to cross-pollinate.

<sup>b</sup> Further selections were made at the end of the season.

## SECOND-GENERATION SELECTIONS

The mortality in the greenhouse planting was very heavy, and only six plants produced seed. Each of the plants produced some seed from self-pollinated blossoms. Certain crosses were made by working a single brush over groups of blossoms on paired plants. Such combinations are designated as 30-12 $\times$ 14B, etc., the number of the pistillate parent being given first in each case and the suffix B being added to distinguish them from crosses in which the flowers of the pistillate plants were emasculated before pollination. The seed produced in such crosses was therefore partly from self-pollination and partly from cross-pollination.

Four of the progenies were tested in 1924, and all but 30-12s were tested in 1925. Three of the selfed lots showed complete resistance, while the other three were clearly heterozygous for resistance. There was also marked segregation in type characters. Progeny 30-12s was planted so late in 1924 that the plants did not mature heads and therefore could not be judged critically for type. There was not sufficient seed for a second trial in 1925, but several plants from the 1924 plantings were saved and grown in an isolated spot in 1925, where they were allowed to become cross-pollinated. Progeny 30-13s, in addition to being completely resistant, was quite uniform in other characters. The color was distinct from that of the majority of the other lots in being yellowish green, approaching that of the standard early Copenhagen Market. The time of maturity appeared to be slightly earlier than the average of the sister progenies and apparently a few days earlier than the Globe line, though it was distinctly later than the earliest strains of Copenhagen Market. The plants were larger and somewhat leafier than the last-named variety, but the heads were typical. (Fig. 4, C, D.) This appeared to be by far the best progeny when resistance and type were both considered. Several third-generation selections were made from it. A trial row of 30-13 $\times$ 15B alongside nonresistant Golden Acre is shown in Figure 3, B. Lot 30-14s showed extreme deviation. The color of the foliage was distinctly more bluish than is the rule, while the heads assumed a distinctly oblong shape. (Fig. 5, D, E, F.) The 1924 trial was planted too late to judge for type, but several plants were saved and seed produced from five in the winter of 1924-25. Progeny 30-15s approached 30-13s, but was not highly resistant and not as uniform in type. Lots 30-16s and 30-17s approached 30-14s as to type characters.

## THIRD-GENERATION SELECTIONS

Of the three progenies selected for continuation, 30-13s gave most promise both for type and resistance. Progenies 30-12s and 30-14s were planted too late in 1924 to judge for type, but retrieval of 30-14s in 1925 showed it to possess certain undesirable qualities. Strain 30-25-A, resulting from 30-12s plants, proved to be highly resistant (Table 2), but since the heads were decidedly flattened it was not carried farther. The progenies from 30-14s (30-30 to 35) showed that in all but one there was, as in the parent progeny, a distinct tendency to produce oblong heads, while segregation was also apparent in color of foliage and in resistance. They were not continued.

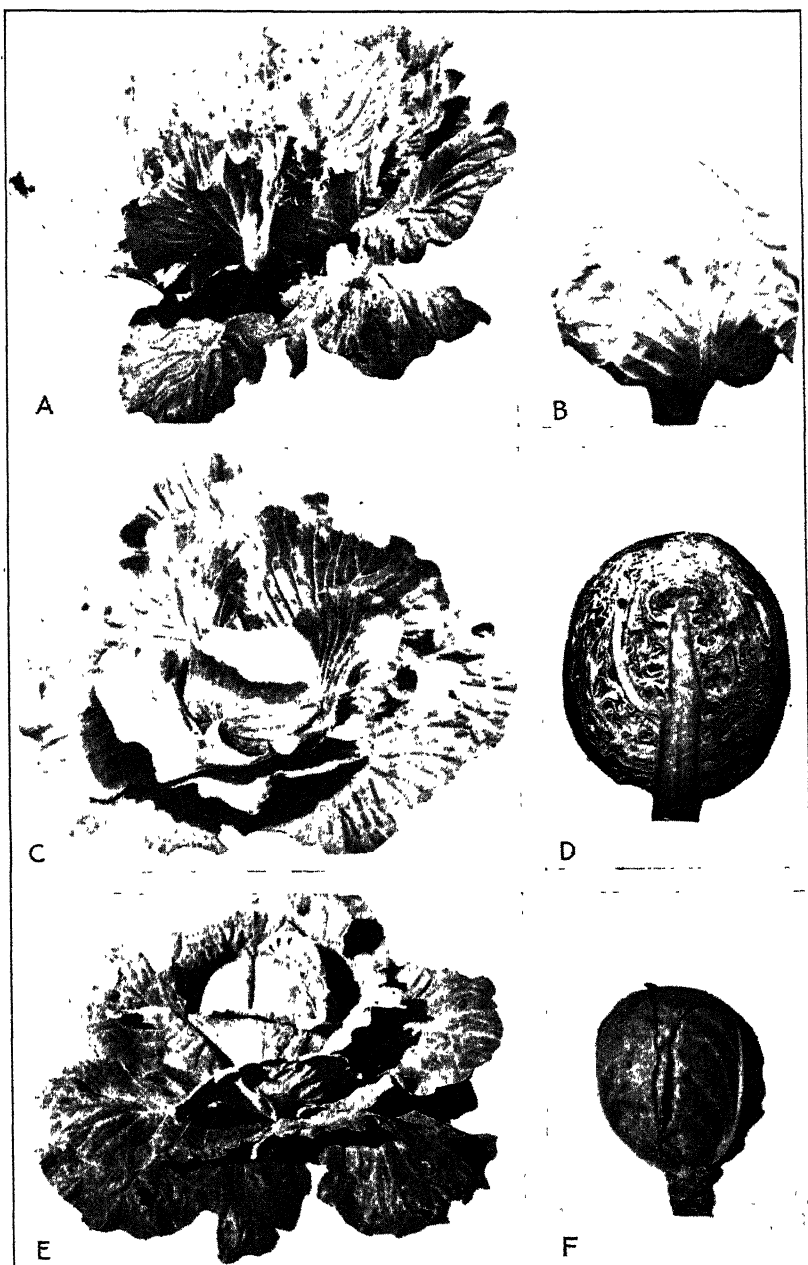


FIG. 5.—Examples of atypical plants which predominated in certain progenies and necessitated disposal of the latter in spite of high resistance. A, All Head Early plant showing long petioles. (Compare with fig. 4, E.) B, pointed head which predominated in a homozygous resistant line selected from All Head Early. (Compare with fig. 4, F.) C, misshapen head common to many progenies from All Head Early. (Compare with fig. 4, E.) D, E, F, three views of elongated head type prevalent in several progenies selected from Copenhagen Market. Note the excessively long core in a head of this type. (Compare with the more desirable type preserved in Marion Market, fig. 4, C, D)

The third-generation progenies derived from 30-13s all showed characters of the parent strains in remarkable uniformity. The yellowish green foliage was prevalent, and the nearly spherical head which flattened very slightly on top with maturity was predominant. Two of the six progenies (30-38s and 30-41s) segregated at the ratio of 3 resistant to 1 susceptible. It is possible that this was the result of pollen contamination of parent plant 30-13, the selfed branches of which were not bagged during the entire period of blossoming. Lots 30-36s and 30-37s were preserved for further seed production.

It is evident from this analysis that the first-generation selections were much improved in degree of resistance. Possibly the crossing of the plants through open-pollination tended to obscure the heterozygous condition as to other characters. The production of selfed progenies in the second generation resulted in a marked segregation both as to resistance and type. By selfing for one more generations it was possible to approach or attain homozygosity for resistance along with greater uniformity of other characters than prevailed in the original stocks. This was particularly true of progenies derived from 30-13s.

#### TYPE AND MULTIPLICATION OF MARION MARKET

Further multiplication of this variety (Marion Market) is to be based upon progenies 30-36s and 30-37s. Plants from these two lots will be used to produce stock seed which may serve as a basis for increase crops. In the meantime preliminary trials on a commercial scale are made possible by seed procured in 1924 from surplus heads selected from progenies 30-1 to 4. Part of this was planted in the trial plots in 1925. Though not as uniform in type as 30-36s or 30-37s, it was quite as good as the parent variety and showed 82 per cent healthy plants and only 14 per cent severely diseased. The remainder of the seed was placed in the Puget Sound seed-growing region in Washington State in 1925 by the National Kraut Packers' Association, and a crop of seed was obtained therefrom in 1926.

The Marion Market variety as represented by progenies 30-36s and 30-37s is a mid-season variety maturing at the same time as or only a few days earlier than the Globe. (See fig. 4, C, D.) The foliage is yellowish green with comparatively little bloom and is very similar in these respects to most strains of Copenhagen Market. The head is spherical or slightly flattened on the top. The stem is medium in length, and the core extends to about the center of the head. The leaves of the head, as in Globe, are unevenly crumpled and not smooth and tightly overlapping.

#### DEVELOPMENT OF THE RESISTANT VARIETY ALL HEAD SELECT FROM ALL HEAD EARLY

##### FIRST AND SECOND GENERATION SELECTIONS

Initial selections were made in 1920 from the surviving plants of All Head Early planted in the trial plot of infested soil. The seed plants were allowed to cross-pollinate in 1921, and in 1922 head-to-row tests were made from five individuals (40-21-1 to 5). None of these showed any marked improvement in resistance over the com-

mercial variety. (Table 3.) One lot (40-21-5) was made the basis of further selection because it contained the largest percentage of plants of desirable type. These second-generation heads were grown for seed in the greenhouse, where the plants were selfed and certain crosses made.

TABLE 3.—*Behavior of first and second generation selections from All Head Early variety of cabbage on yellows-infested soil*

First-generation selections, trial in 1922			Second-generation selections, trial in 1923		
Progeny No. <sup>a</sup>	Total number of plants	Yellows (per cent)	Progeny No. <sup>b</sup>	Total number of plants	Yellows (per cent)
40-21-1.....	157	75	40-1s.....	85	70
40-21-2.....	231	69	40-1×6.....	65	0
40-21-3.....	245	66	40-1×7.....	51	15
40-21-4.....	263	64	40-4s.....	20	10
40-21-5.....	265	71	40-4×6.....	18	0
			40-4×7.....	31	18
			40-6×7.....	14	0
			40-7s.....	53	20
			40-7×3.....	25	15
			40-7×6.....	39	0
Commercial All Head.....	105	77		213	90
Wisconsin All Seasons.....	364	8		258	1

<sup>a</sup> The parent plants of these progenies were allowed to cross-pollinate.

<sup>b</sup> See Table 2 for explanation of progeny numbers.

<sup>c</sup> Further selections continued.

The second-generation progenies (Table 3) showed much improvement in resistance; 40-4s and 40-7s appeared to segregate roughly at the ratio of 3 resistant to 1 susceptible, indicating that the plants were heterozygous for resistance. Wherever plant 40-6 entered into crosses the progeny was completely resistant, indicating that it was homozygous for resistance. Unfortunately, no selfed progeny from this plant was obtained. Segregation for type characters was also evident. Lots 40-4s and 40-4×6 were of the most satisfactory type. Progeny 40-7s and crosses in which 40-7 was one of the parents were not true as to color and showed a tendency to produce misshapen heads. Further selection from several progenies was continued, as indicated in Table 3.

#### THIRD-GENERATION SELECTIONS

The results of trials of the third-generation progenies on infested soil in 1924 and 1925 are given in Table 4. A perusal of these data shows that several progenies were obtained which appeared to be highly resistant to yellows. Thus lots 40-93s, 40-120s, 40-130s, and crosses in which 40-130 served as one parent, 40-137s, 40-199s, and most of the crosses in which 40-80 entered as a parent, showed very few diseased plants. Most of them, however, showed one or more undesirable qualities, such as off color, too long petioles, misshapen heads, or late maturity. Some of these undesirable characters are illustrated in Figure 5. Certain other progenies, not so highly resistant, were much superior in type, of which the most promising were 40-76s, 40-91s, 40-91×93, and 40-96s.

TABLE 4.—*Behavior of third-generation selections from All Head Early variety of cabbage on yellows-infested soil*

Parent strain	Progeny No. <sup>a</sup>	1924 trials			1925 trials		
		Total number of plants	Yellows, dead or severe (per cent)	Yellows, slight or re-covered (per cent)	Total number of plants	Yellows, dead or severe (per cent)	Yellows, slight or re-covered (per cent)
40-4s	40-75s	29	17	27			
	40-76s	8	0	<sup>b</sup> 12			
	40-80×76	9	0	0			
	40-80×79	15	0	0			
	40-80×81	29	0	0			
	40-80×82	51	0	2	23	0	0
	40-82×80	41	2	12	47	0	0
40-4×6	40-90s	14	14	29			
	40-91s	45	7	<sup>b</sup> 9			
	40-91×93	74	4	<sup>b</sup> 11	50	0	<sup>b</sup> 6
	40-93s	19	21	0			
40-4×7	40-96s	24	13	<sup>b</sup> 17			
	40-97s	19	0	11			
40-6×7	40-112s	16	6	25			
	40-114s	14	0	7			
	40-115s	12	0	38			
	40-120s	11	0	0			
40-7s	40-131s	62	19	55	51	14	17
	40-137s	82	0	<sup>b</sup> 0	50	0	2
	40-130s	31	3	0			
40-7s and 40-4s	40-130×75	61	0	0			
	40-130×79	28	0	0			
	40-130×82	36	0	0			
40-7×6	40-199s	36	0	11	49	0	0
	40-207s	18	19	19			
	40-212s	32	0	19			
Commercial All Head							
Early		23	95	0	112	54	46
Wisconsin All Seasons		55	0	2	50	0	4

<sup>a</sup> See Table 2 for explanation of progeny numbers.<sup>b</sup> Further selections continued.

Inasmuch as the aim in this improvement work was to procure strains acceptable as to type as well as resistance, it was not considered advisable to continue those progenies which showed distinctly undesirable qualities even though they were highly resistant. All of these except 40-137s are therefore dropped from further consideration in this discussion, even though certain of them were continued for other purposes. Progeny 40-137s, though slightly off in certain respects, was continued. The major emphasis was turned to further selection from the somewhat less resistant lots 40-76s, 40-91s, 40-91×93, and 40-96s. From these, fourth-generation trials were made in 1925 and 1926.

#### FOURTH AND FIFTH GENERATION SELECTIONS

Most of the fourth-generation selections were grown to seed in the greenhouse in the winter of 1924-25 and the first trials made in 1925. Some additional selections from 40-91×93 were made in the summer of 1925, and the new progenies thus obtained were not tested until 1926. A summary of the results of these trials is given in Table 5. It will be seen that the lots derived from 40-137s continued to fall in the highly resistant class. They were not improved

in type, however, over the parent progeny. Of those derived from the less resistant lots (40-76s, 40-91s, 40-91×93, and 40-96s), some were no more resistant than the parent stocks, while others showed little or no disease. In general, the better qualities of the parent strains were preserved. In 1925 further selections were made from 40-303×353, and 40-326s. The trials of the resulting progenies are given in the same table. While they were not all free from disease, all but 40-512s and 40-538s showed only slight traces of yellows.

At the end of the 1926 season a number of families were on hand which were highly resistant to yellows and at the same time conformed very uniformly to the desired type. The best strains were those which were derived originally from 40-4×6 (Table 3) and then from 40-91×93. (Table 4.) Certain of these were of the fourth generation and others of the fifth generation. As they had been subjected to inbreeding for these several generations, the expected reduction in vigor was evident. Further pure-line selection might bring further reduction and thus offset the gain acquired by improved uniformity in type and resistance. At the end of the 1926 season, therefore, four progenies were selected for their combination of good type with resistance. There were two fourth-generation selections (40-406s and 40-406×407) and two fifth-generation selections (40-508×512 and 40-511×510). A portion of each of these is being grown in mixed plantation in 1927 to produce stock seed for multiplication.

It is hoped that this combination, even of closely related progenies, will result in restoring any loss of vigor which became apparent in the fourth and fifth generations as a result of selfing. As a safeguard against the possibility that such a combination of strains will not sufficiently restore loss of vigor, a portion of the heads will be planted with heads selected from 40-535s. The latter is derived by a line of selection distinct from that of the others. It is highly resistant and not greatly removed from the other strain in type.

#### TYPE AND MULTIPLICATION OF ALL HEAD SELECT

As already pointed out, the four best strains from the 1926 trials are to be combined to produce stock seed in 1927. This seed will serve as a basis for multiplication. In the meantime the urgent demands for seed of a resistant strain of this type justified steps to multiply some of the less completely finished progenies. Accordingly, about 100 heads were selected from the trials of 1923 (Table 3), and from them a quantity of stock seed was grown in 1924. When tested in 1925 it proved to be highly resistant and fairly uniform in type. Most of this lot was placed for seed growing in the Puget Sound region in 1925 by the National Kraut Packers' Association, and a crop of seed was obtained in 1926. Similarly, the best heads not taken for special purposes from the 1924 trials (Table 4) were grown for stock seed in 1925 and a second multiplication crop started in 1926. These advance strains of the variety All Head Select will be available for general use until the improved selections of 1926 can be multiplied.

The type of All Head Select as represented in strains 40-406s, 406×407, 508×512, and 511×510 is very similar to that of 40-91×93 shown in Figure 4, E, F. The strain is very similar to the original All Head Early. The stem is short, the head fairly flat, and

the color of foliage intermediate between the yellowish green of Copenhagen Market and the bluish green of Glory of Enkhuizen. The outer leaves at maturity are comparatively few and the petioles very short, giving a compact plant with a fairly prominent head. It matures about the same time as the parent variety and close to Globe and Marion Market. It is two weeks or more ahead of Wisconsin All Seasons or Wisconsin Brunswick and thus supplements the last two varieties in season.

TABLE 5.—Behavior of fourth and fifth generation selections from All Head Early variety of cabbage on yellows-infested soil

Generation	Parent strain	Progeny No. <sup>a</sup>	1925 trials			1926 trials						
			Total number of plants	Yellows, dead or severe (per cent)	Yellows, slight or recovered (per cent)	First planting			Second planting			
						Total number of plants	Yellows, dead or severe (per cent)	Yellows, slight or recovered (per cent)	Total number of plants	Yellows, dead or severe (per cent)	Yellows, slight or recovered (per cent)	
Fourth	40-76s	40-302s	24	0	25							
		40-302×306	24	8	4							
		40-302×307	26	0	20							
		40-303s	16	0	b 0							
		40-303×353	15	0	b 7							
		40-303×304	12	0	0							
		40-303×310	25	0	0							
		40-304×310	25	12	0							
	40-91s	40-305s	28	7	11							
		40-308s	50	0	4							
		40-309s	25	0	4							
		40-308×309	27	4	11							
	40-91×93	40-310s	25	0	8							
		40-325s	15	0	20	36	6	3				
		40-325×326B							25	0	0	
		40-326s	15	0	b 0	18	0	6				
		40-326×329B				40	0	3				
		40-329s	21	0	10							
		40-329×326B	25	0	16							
		40-402s			20	0	0					
		40-403s			7	0	0					
		40-405s			33	0	6		26	4	0	
		40-406s			17	0	0		24	0	0	
		40-406×407			64	0	9		25	0	0	
		40-407s			34	0	0					
		40-408s			20	0	20					
		40-410s			19	0	0		25	0	16	
		40-410×411			32	0	6					
	40-96s	40-411s							25	0	0	
		40-334s	30	0	0							
		40-335s	27	0	0							
		40-137s	40-353s	24	0	4						
40-354s			33	0	3							
40-355s			34	3	0							
40-421s									23	0	0	
40-426s								25	0	0		
40-326s	40-508×512			16	0	0		54	0	7		
	40-511s			9	0	11						
	40-511×510			48	0	0		61	0	0		
	40-512s			15	7	7						
40-303×353	40-535s							23	0	0		
	40-538s							21	5	0		
Commercial Wisconsin All Seasons			215	51	34	51	61	14	240	61	27	
			100	0	6	74	0	5	23	0	0	

\* See Table 2 for explanation of progeny numbers.

b Further selections continued.

## DISCUSSION

The results of several seasons' experimentation support the earlier supposition that there exists in most if not all varieties of cabbage some degree of natural resistance to the *Fusarium* disease, and that by selection of individuals which survive upon infested soil, resistant strains may be readily developed. Results with the development of the three new mid-season varieties emphasize the necessity of pure-line selection, and it is only by such methods that one may hope to fix resistance in a given strain. It appears that resistance as expressed under field conditions in southern Wisconsin is governed by a single dominant factor, and thus homozygous lines may be expected with two or three generations' selections.

The danger of digression in type during the progress of selection for resistance is equally evident. There is little to support the theory that resistance is linked with important type characters. On the other hand, most commercial cabbage varieties are more or less heterozygous for various characters, and segregation is commonly found when pure-line selections for resistance is started. This may appear disadvantageous, but it likewise may be beneficial, inasmuch as it offers the opportunity to reject undesirable qualities which might otherwise be masked because of their recessive nature. The results show that if one so desires he may by this means improve the uniformity in type of a given strain at the same time that he is selecting for resistance. It is certain that selection for resistance alone may defeat its own end by so changing the type through indiscriminate perpetuation of undesirable individuals as to reduce the commercial value of the resulting strain. The danger of reduction in vigor from repeated selfing imposes a limitation to indefinite perpetuation of selfed lines. It may be advantageous in some cases, as has been suggested with All Head Select, to carry parallel but distinct selfed lines which may be intercrossed eventually when each has reached a desirable degree of uniformity.

The three mid-season varieties All Head Select, Marion Market, and Globe have been advanced to a high degree of resistance and of uniformity of type. They appear to be nearly if not completely homozygous for resistance under the average field environment in which they were selected. It has been shown by Tisdale (7) that the resistance of Wisconsin Hollander may be broken down when plants are grown at high constant soil temperatures under greenhouse conditions, and similar results were attained when seed was sown in a naturally infested field during midsummer. In certain commercial cabbage sections the plants are probably exposed to a higher temperature at an earlier stage in their development than in southern Wisconsin. The period of exposure to favorable conditions for the disease may therefore be longer and the extreme temperatures may be higher. Just how much these varied environments will inhibit the commercial value of these new varieties remains to be determined through repeated testing by those who may be interested in their use. Variation in the causal organism, especially with respect to its selective pathogenicity, must also be looked upon as a possible factor influencing their behavior in different localities.

Confidence in the usefulness of these new varieties in latitudes south of Wisconsin is based upon field trials carried on in cooperation

with pathologists of other States. These have included critical tests adjacent to susceptible commercial strains on yellows-infested soil in northern and central Indiana, northern Ohio, Maryland, southwestern Virginia, western Tennessee, southeastern Iowa, and northeastern Kansas. In all cases these trials followed the period and methods used for the commercial crop in each locality. With the exception of the Muscatine, Iowa, trials of 1926, where the incidence of yellows was somewhat higher than in the Kenosha plot,<sup>6</sup> the three varieties stood up as well in other localities as they did in southeastern Wisconsin. It is believed, therefore, that they will usually prove satisfactory for commercial use on infested soil.

### SUMMARY

The main purpose of the present paper is to report the essential details which concern the development of three new *Fusarium*-resistant varieties of cabbage, all of which are classified in the mid-season group.

The methods of selection, hybridization, and field testing are described.

The Globe variety is a selection from Glory of Enkhuizen, a commercial variety of the round-head type. After three generations' selection, progenies were obtained which resemble the parent variety in its main features and were perfectly free from signs of yellows in the 1926 trials.

In like manner the Marion Market was brought to the same degree of resistance from original selections out of Copenhagen Market, a commercial variety of the round-head type. The type, though distinct from some of the early-maturing strains of the parent variety, is well suited for use as a mid-season type.

All Head Select was selected from the flathead commercial variety All Head Early. This strain has given the greatest difficulty in securing the combination of desirable type and resistance. Certain fourth and fifth generation progenies are satisfactory and are to be multiplied.

In all three varieties the trials of 1926 offered the most advanced and satisfactory selections. These will be multiplied for commercial use. In the meantime certain less-advanced progenies of each variety have been multiplied by the National Kraut Packers' Association and are now available for general use.

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<sup>6</sup> Since the preparation of this manuscript, results of a trial in 1927 with Marion Market on thoroughly yellows-infested soil at Muscatine, Iowa, showed it to stand up quite as well as in the Kenosha plots, while the susceptible strains in both localities were badly diseased.

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# BACTERIAL STRIPE BLIGHT OF OATS<sup>1</sup>

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## INTRODUCTION

In the issue of *Phytopathology* for September, 1918,<sup>3</sup> a "stripe blight" of oats was mentioned by the writer as occurring in Wisconsin and adjoining States. The signs of the disease were described as distinctly different from those of halo blight of oats<sup>4</sup> but the causal organism was not named or described. Since that time specimens of this leaf disease have been sent in from various parts of the United States, and the causal organism has been studied in its relation to *Bacterium coronafaciens* Elliott and to the ability of the two organisms to infect various varieties of oats and other Gramineae.

## SIGNS OF THE DISEASE

The lesions first appear as sunken, water-soaked dots, which, when very numerous, cause the collapse of the leaf tissue, or, when coalescing, form long water-soaked stripes (fig. 1) or blotches which may show narrow yellowish margins. Under moist conditions, especially early in the morning, bacterial exudate stands out in drops along the lesions. This exudate dries down to thin white scales, and the lesions as they become older turn a rusty translucent brown. The streaks vary in length from a fraction of an inch to several inches or may extend the length of the leaf blade and down to the sheath. Halo-like borders do not develop about these lesions.

Lesions may occur on plants in all stages of development from seedlings to mature plants. While the lesions are found chiefly on the leaves, occasionally they occur on sheaths, culms, pedicels, and glumes. Sometimes the entire top of a plant is killed by the water-soaked lesions which spread over the culm and the sheath, inclosing the panicle. The long, water-soaked, stripelike lesions, the absence of a halo, and the presence of exudate serve to distinguish stripe blight from halo blight of oats. (Fig. 2.)

## GEOGRAPHICAL DISTRIBUTION

Stripe-blight lesions were first observed and infected leaves were collected by A. G. Johnson at Urbana, Ill., and Wooster, Ohio, during the season of 1917. From that year to the spring of 1926,

<sup>1</sup> Received for publication June 16, 1927; issued December, 1927.

<sup>2</sup> The writer is indebted to A. G. Johnson, of the Office of Cereal Crops and Diseases, for collecting diseased plants and for helpful suggestions.

<sup>3</sup> ELLIOTT, C. BACTERIAL OAT BLIGHT. *Phytopathology* 8: 489-490. 1918.

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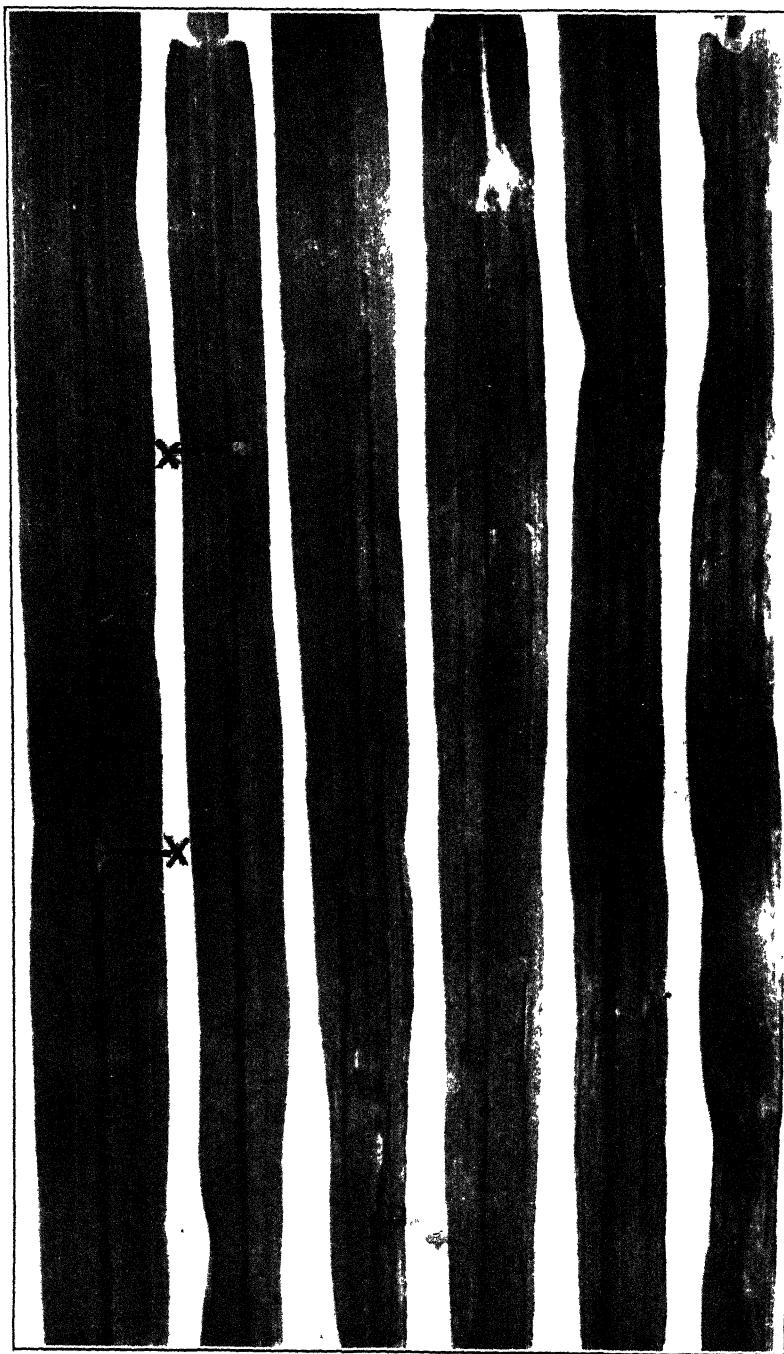


FIG. 1.—Stripe-blight lesions and exudate (X) from natural infections on leaves of Wisconsin No. 146 oat (*Avena byzantina* C. Koch) collected at Madison, Wis., June 13, 1918.  $\times 2$ .

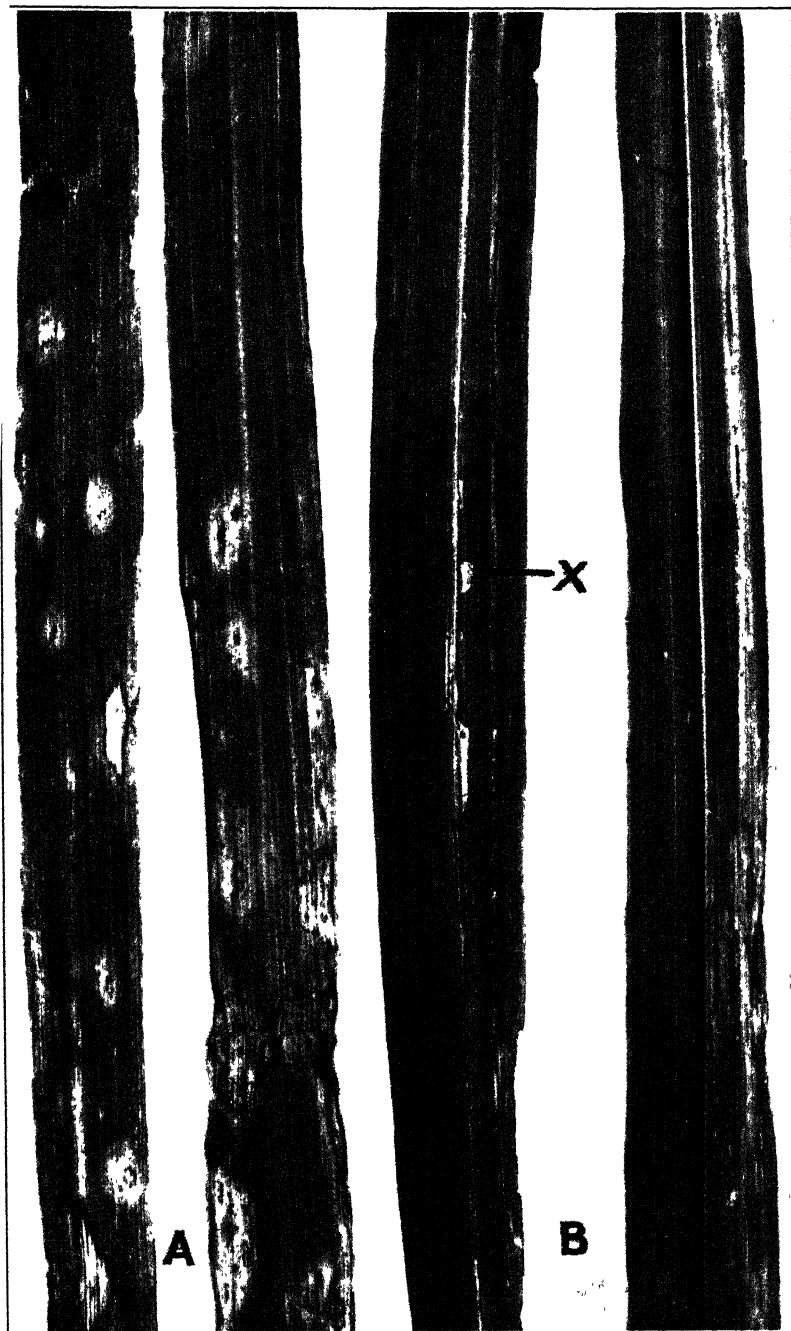


FIG. 2.—Halo-blight lesions (A) and stripe-blight lesions (B) from artificial inoculations with *Bacterium coronafaciens* (isolation No. 74) and with *Bact. striafaciens* (isolation No. 10), respectively, on leaves of Wisconsin Pedigree No. 14 oat (*Avena sativa*). Photographed October 11, 1924. Natural size

oat leaves containing lesions of stripe blight have been collected in different parts of the country, in almost every year, and on plants from the seedling stage to those nearing maturity. Table 1 shows the localities from which specimens have been received and the varieties infected.

TABLE 1.—*Collections of stripe blight of oats, showing oat varieties, dates and localities of collection, and names of collectors*

Variety	Date	Locality	Collector
Swedish Select.....	June 20, 1917	Urbana, Ill.....	A. G. Johnson.
Not known.....	June 21, 1917	La Fayette, Ind.....	Do.
Wideawake.....	June 23, 1917	Wooster, Ohio.....	Do.
Not known.....	July 14, 1917	Custer, S. Dak.....	C. W. Hungerford.
Wisconsin No. 146 (Avena byzantina).....	June 10, 1918	Hill farm, Madison, Wis.....	Charlotte Elliott.
Not known.....	June 15, 1918	College Hill, Madison, Wis.....	Do.
Wisconsin No. 146 (Avena byzantina).....	.....do.....	.....do.....	Do.
Not known.....	June 25, 1918	Wooster, Ohio.....	A. D. Selby.
Wisconsin Pedigree No. 14.....	July 17, 1918	Madison, Wis.....	Charlotte Elliott.
Not known.....	Mar. 29, 1919	Davis, Calif.....	A. G. Johnson.
Do.....	Apr. 2, 1919	.....do.....	Do.
Red Rustproof (Avena byzantina).....	June 7, 1920	Granite City, Ill.....	A. G. Johnson, H. B. Humphrey, and H. H. McKinney.
Not known.....	.....do.....	Madison, Wis.....	Charlotte Elliott.
Wisconsin No. 103.....	June 9, 1920	.....do.....	Do.
Not known.....	June 28, 1920	Hill farm, Madison, Wis.....	Do.
Sixty-Day.....	May 26, 1921	Mandan, N. Dak.....	A. G. Johnson.
Big Four and Silvermine.....	June 18, 1924	La Fayette, Ind.....	A. G. Johnson and E. B. Mains.
Silvermine.....	June 21, 1924	De Kalb, Ill.....	C. S. Reddy and A. G. Johnson.
Not known.....	.....do.....	Chenao, Ill.....	A. G. Johnson.
Do.....	.....do.....	Bloomington, Ill.....	C. S. Reddy and A. G. Johnson.
Do.....	June 22, 1924	Oregon, Wis.....	A. G. Johnson.
Do.....	June 23, 1924	Madison, Wis.....	Do.
C. I. 620 (unnamed).....	June 26, 1924	University farm, St. Paul, Minn.....	J. J. Christensen and A. G. Johnson.
Black Tartarian.....	.....do.....	St. Paul, Minn.....	A. G. Johnson and O. S. Aamodt.
Guyra.....	Apr. 30, 1925	Davis, Calif.....	A. G. Johnson.
White Ligowa.....	May 1, 1925	.....do.....	Do.
Red Rustproof [Texas Red, Calif. No. 1003 (Avena byzantina)].....	.....do.....	.....do.....	Do.
Garton Yellow, C. I. No. 1612.....	May 9, 1925	Corvallis, Oreg.....	Do.
Belyak, C. I. No. 1899.....	.....do.....	.....do.....	Do.
Markton, C. I. No. 2053.....	.....do.....	.....do.....	Do.
Black Mesdag, C. I. No. 1877.....	May 12, 1925	Pullman, Wash.....	E. F. Gaines and A. G. Johnson.
Not known.....	Apr. 20, 1926	Davis, Calif.....	W. W. Mackie.

## THE CAUSAL ORGANISM

### ISOLATIONS

From stripe-blight lesions, collected on several oat varieties from different parts of the country, more than 40 isolations have been made. The organism is not sensitive to sterilization. The leaf tissue may be dipped in 95 per cent alcohol, put into mercuric chloride solution (1:1,000) for two minutes, washed, and then crushed in broth, from which the plates are poured. While yellow colonies were observed on some of the plates, the characteristic white colonies were much more numerous and often appeared in pure culture. In many cases plates were poured from halo and stripe lesions at the same time. The white colonies developing from both types of

lesions were always so nearly alike that they could not be distinguished. Colonies begin to appear on the plates after about 48 hours. They are raised, white, smooth, shining, and round or with slightly undulate thin margins. (Fig. 3, D.) The narrow radiate border shown in Figure 3, C, is characteristic, especially of isolation No. 66b. There also are more or less well-developed internal fish-scale markings similar to those of halo-blight colonies.

#### INOCULATIONS

Inoculations were made by spraying oat plants with water suspensions of the organisms from young agar slants. The plants were then kept in damp chambers for about 48 hours. Controls were sprayed with sterile water and kept under similar conditions. Halo-blight organisms were used for comparison in the same inoculation experiments with the stripe-blight organisms, and they always produced abundant typical halo lesions. Infections with both organisms began to appear in from three to five days. The lesions of stripe blight first appeared as water-soaked dots, 1 millimeter in diameter, which turned brown with translucent centers and sometimes had narrow yellow margins. These centers of infection increase until they are 1 to 2.5 centimeters in length, or they sometimes may extend one-half to three-fourths the length of the leaf. The long narrow lesions are brown and water-soaked, with narrow yellow-brown margins. Where lesions are very numerous, the whole leaf turns yellow, with points of infection evident, and then collapses. Small dried scales of exudate are apparent at points of infection.

Thirty-five inoculations with the stripe organism were made in the greenhouse and 16 in the field. Of the 35 greenhouse inoculations 5 produced no lesions, owing to unfavorable conditions of temperature and moisture or to resistant varieties of oats. Of the 30 successful inoculations, 8 produced only scattered but typical lesions and the remainder moderate to heavy infection.

The lesions produced by these inoculations always were typical of stripe-blight lesions. (Fig. 2, B.) No halo-blight lesions ever developed from inoculations with the stripe-blight organism, although typical halo-blight lesions were produced on the same varieties inoculated at the same time with the halo-blight organism. (Fig. 2, A.) In the field it is more difficult to control environmental conditions, and so the results of inoculations are less satisfactory. Of the 16 field inoculations, 6 were negative and 10 positive, only 2 of the latter showing heavy infection.

Table 2 shows the results of one inoculation experiment which was made both in the greenhouse and in the field in the spring of 1926. Ninety-two varieties and strains, including most of the cultivated species and varieties of oats, were sprayed with water suspensions of both halo and stripe organisms, to learn whether there would be any varietal differences in susceptibility to the two organisms. In all varieties the susceptibility to the two organisms was nearly or quite the same, so that the organisms could not be separated on this basis. Where there were some differences in host reaction in the greenhouse, a second set of inoculations was tried, but these only confirmed the previous conclusion that each variety reacts in about the same way to both organisms.

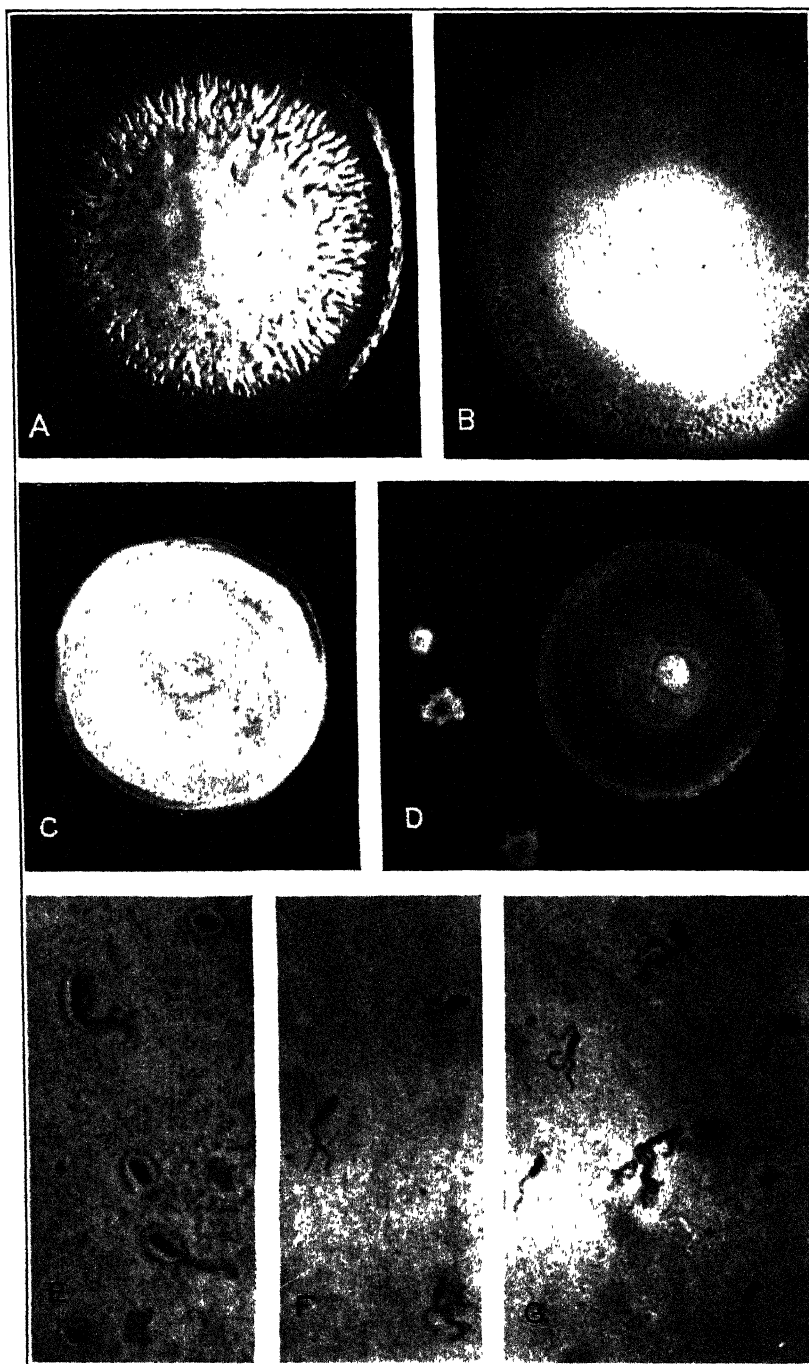


FIG. 3.—A, *Bacterium striaefaciens*, 6-day colony of isolation No. 10 on Thaxter's potato agar. Photographed by direct light Oct. 17, 1924.  $\times 5$ . B, *Bact. coronafaciens*, 6-day colony on Thaxter's potato agar. Photographed by direct light Oct. 17, 1924.  $\times 5$ . C, *Bact. striaefaciens*, 3-day colony of isolation No. 66b on +15 beef-peptone agar. Photographed by transmitted light.  $\times 5$ . D, *Bact. striaefaciens*, 18-day colony of isolation No. 66b on +15 beef-peptone agar. Photographed by transmitted light.  $\times 6$ . E, *Bact. striaefaciens* from 3-day culture of isolation No. 14, grown on beef-peptone agar and stained by Casares-Gil carbol-fuchsin method, showing capsules.  $\times 1,700$ . F and G, *Bact. striaefaciens* from 24-hour culture of isolation No. 66b grown on beef-peptone agar and stained by Casares-Gil carbol-fuchsin method, showing flagella.  $\times 1,550$

TABLE 2.—Degrees of infection of 92 varieties and strains of oats grown in the greenhouse and in the field at Arlington Experiment Farm, Rosslyn, Va., and inoculated with the organisms causing halo blight and stripe blight, respectively, to determine any differences in susceptibility to the two organisms

[Degrees of infection: 0=None, 1=slight, 2=moderate, 3=heavy, 4=very heavy]

Species and variety	C. I. No.	Degree of infection					
		In greenhouse				In field June 8	
		Feb. 5		Feb. 19		Halo blight	Stripe blight
		Halo blight	Stripe blight	Halo blight	Stripe blight		
<i>Avena byzantina</i> :							
Coastblack.....	1025	0	0	2	0	1	1
Black Algerian.....	840-1	1	2			1	1
Sterilis Selection (of Etheridge).....	1859-1	0	1			1	1
Red Rustproof.....	1039	1	2			0	1
Red Rustproof (of Etheridge).....	1860	1	0			0	0
Fulghum.....	708	0	0	0	0	0	0
Burt (of Etheridge).....	1861	1	1			0	1
Sunrise (Calif. No. 1012).....	1799	1	2			1	1
Culred.....	518-189	1	1			1	1
<i>Avena sativa</i> :							
Boswell.....	480	2	2			0	0
Black Winter.....	608	0	3	1	2	1	1
Hatchett.....	838	0	1			1	1
Tech.....	905	2	2			1	1
Winter Turf.....	1570	2	0			0	0
Custis.....	2041	2	1			1	1
Lee.....	2042	2	1			1	1
Culberson.....	273	0	0	1	0	0	0
Dwarf Culberson.....	748	0	0	1	0	1	1
Bicknell.....	206-151	1	1			2	2
Black Mogul.....	1074	2	4			1	1
Black Bell.....	1767	2	3			0	1
Victor.....	803	2	3			0	1
Black Mesdag.....	1877	2	3			1	1
Monarch.....	1876	1	1			1	1
Joanette (of Etheridge).....	1880	1	2			1	1
Early Joanette.....	1092	1	2			1	1
Colburt.....	2019	0	0	0	0	1	0
Black Diamond.....	1878	0	2			1	1
Monarch Selection (of Etheridge).....	1879	1	2			2	2
Old Island Black.....	1756	2	2			1	1
Do.....	2035	2	1				
North Finnish.....	1882	3	2			1	1
Ruakura.....	2025	1	0			1	1
Cornellian.....	1242	1	0			0	0
White Maine.....	1692	3	3			1	2
Richland.....	787	3	3			2	2
Kherson.....	1148	1	2			2	2
State Pride.....	1154	3	3			1	0
Navarro (Ferguson Navarro).....	966	0	0	0	0	0	0
Markton.....	2053	3	3			2	2
Madrid.....	603	3	3			1	1
Aurora.....	831	0	0	0	0	0	0
Golden Drop.....	1890	3	2			1	1
Green Russian.....	1978	3	1			0	0
Iogren.....	2024	1	1			1	1
Japan.....	1157	4	1			0	1
Minota.....	1285	1	0			1	1
Awless Probsteler.....	1888	2	1			1	0
Probsteler.....	1876	3	2			2	1
June (of Etheridge).....	1902	3	1			1	1
Wernich Golden.....	1746	2	1			1	1
Garton No. 473.....	1613	2	2			1	1
Albion.....	729	3	2			1	1
Cole.....	834	3	3			1	0
Sixty-Day.....	1887	1	1			1	0
Iowar.....	847	3	1			1	0
Early Ohio.....	1117	3	3			2	1
Early Champion.....	1623	3	2			1	1
Daubeney.....	1621	1	1			1	1
Yakutsk.....	498	1	1			1	0
White Bonanza.....	1686	1	0	0	0	1	1
Canadian.....	1625	1	1			0	0
Castleton Potato.....	1972	2	1			1	1

TABLE 2.—Degrees of infection of 92 varieties and strains of oats grown in the greenhouse and in the field at Arlington Experiment Farm, Rosslyn, Va., and inoculated with the organisms causing halo blight and stripe blight, respectively, to determine any differences in susceptibility to the two organisms—Continued

[Degrees of infection: 0=None, 1=slight, 2=moderate, 3=heavy, 4=very heavy]

Species and variety	C. I. No.	Degree of infection					
		In greenhouse				In field June 8	
		Feb. 5		Feb. 19			
		Halo blight	Stripe blight	Halo blight	Stripe blight	Halo blight	Stripe blight
<i>Avena sativa</i> —Continued.							
Early Mountain.....	1624	0	1	.....	.....	1	1
Tobolsk.....	1709	1	1	.....	.....	0	0
Wisconsin Wonder.....	1645	2	1	.....	.....	0	0
Silvermine Selection (of Etheridge).....	1894	.....	.....	.....	.....	0	0
White Avalanche.....	1440	0	0	.....	.....	1	1
Irish Victor.....	1896	2	1	.....	.....	1	1
O. A. C. No. 72.....	846	1	1	.....	.....	1	1
Scottish Chief.....	1699	1	1	.....	.....	1	1
Gothland.....	1898	3	3	.....	.....	2	1
Minnesota No. 295.....	1290	1	2	.....	.....	0	0
Danish Island.....	1684	1	1	.....	.....	1	1
Pringle Progress.....	1717	2	1	.....	.....	1	1
Belyak.....	1630	2	1	.....	.....	2	1
Swedish Select.....	1375	0	0	1	1	0	0
Colorado No. 37.....	1640	1	0	.....	.....	0	1
Lincoln.....	1262	1	0	.....	.....	1	0
Silvermine.....	1013	2	0	.....	.....	1	0
Comewell.....	1317	1	1	.....	.....	1	1
Victory.....	1145	0	0	2	2	1	0
Tabor.....	1777	1	2	.....	.....	1	0
<i>Avena sativa orientalis</i> :							
Storm King.....	1602	2	2	.....	.....	1	1
Tartar King.....	1599	2	1	.....	.....	1	1
Marvelous.....	1999	0	2	.....	.....	1	2
Green Mountain.....	1872	0	3	3	1	1	1
White Tartar.....	1614	1	1	.....	.....	0	1
<i>Avena nuda</i> :							
Hoejer (Hull-less).....	1003	0	0	0	0	.....	0
Kilby (Hull-less).....	1995	1	0	.....	.....	.....	0
Liberty (Hull-less).....	845	1	2	.....	.....	.....	.....
Fowlds (Hull-less).....	1996	0	1	.....	.....	.....	.....
Hull-less.....	1770	0	1	.....	.....	.....	.....

Inoculations were made on *Bromus inermis* Leyss, *B. carinatus* Hook and Arn., *Agropyron repens* (L.) Beauv., and *Chaetochloa lutescens* (Weigel) Stuntz (*Setaria glauca* of authors). No lesions were produced on any of these hosts, although inoculations on oats at the same time produced definite stripe-blight and halo-blight infections.

Inoculations with the stripe-blight organism (66b) were made on tobacco, cauliflower, cabbage, and broomcorn, but with negative results.

Inoculations with the stripe-blight organism (isolation No. 14) and the halo-blight organism (No. 75) were made on *Avena strigosa* Schreb. and Markton oats and also on unidentified varieties of wheat, rye, and barley in the greenhouse. Barley and oats showed slight infection with the stripe-blight organism and oats and rye with *Bacterium coronafaciens*.

## COMPARISON WITH OTHER ORGANISMS

H. R. Rosen in 1919<sup>5</sup> and 1922<sup>6</sup> described a bacterial disease of foxtail (*Chaetochloa lutescens*), the causal organism of which he said was infectious to many Gramineae, including oats, on which the lesions were not unlike those produced by *Bacterium coronafaciens*. The spots on oats, he says, may vary from indefinite light-yellow areas to withered grayish green with marked tinges of red when the infected areas coalesce. Ordinarily there is no exudate. Infections by *Bact. coronafaciens* on oats show no exudate, but are definite oval yellow halos about sunken tissue at the points of infection. The lesions produced on oats by the foxtail organism are evidently quite different from the water-soaked dots and translucent brown streaks of the stripe organism. The occurrence of abundant exudate under favorable conditions also is a distinguishing feature.

The stripe-blight organism differs from *Pseudomonas alboprecipitans* in important morphological and cultural characters, as recorded by Rosen. The writer was not able to obtain a culture of the foxtail organism for comparative cultural and inoculation tests.

According to Reddy and Godkin,<sup>7</sup> the brome-grass organism, *Bacterium coronafaciens* var. *atropurpureum*, differs from *Bact. coronafaciens* only in host reactions. The latter oat organism infected only one species of brome grass (*Bromus carinatus*), while the brome-grass organism infects both brome grass and oats. The kinds of lesions produced on oats by the two organisms are not identical. Those produced by the brome-grass organism have a more water-soaked appearance and narrower halos than those produced by the oat organism. On *B. inermis* the lesions are at first circular to elliptical water-soaked areas with yellowish green halos. Later these spots become dark chocolate or purplish brown and usually are linear. Similar lesions occur on *Agropyron repens* (L.) Beauv., on which they are dark purplish brown to black. From this description of the lesions it is evident that those caused by the brome-grass organism are distinctly different from the water-soaked dots and streaks produced by the oat-stripe organism.

## CULTURAL AND MORPHOLOGICAL CHARACTERS

Through all the tests on culture media the stripe-blight organism and *Bacterium coronafaciens* were carried along together, but only slight differences were observed.

In dimensions, the stripe-blight organism was consistently smaller, having average measurements of 0.66 by 1.76  $\mu$ . *Bacterium coronafaciens* grown at the same time and under the same conditions and stained in the same way had average measurements of 0.68 by 2.40  $\mu$ . The organisms were grown for one or two days on beef-peptone and potato agar and were stained with gentian violet.

Both organisms have capsules (fig. 3, E) and one to several polar flagella (fig. 3, F). They are Gram-negative and not acid fast. Growth on beef-peptone agar is white and slightly raised, with entire to slightly undulate margins and internal fish-scale markings. Agar

<sup>5</sup> ROSEN, H. R. A PRELIMINARY NOTE ON A BACTERIAL DISEASE OF FOXTAIL. Science 49: 291. 1919.

<sup>6</sup> ROSEN, H. R. A BACTERIAL DISEASE OF FOXTAIL (*CHAETOCHELOA LUTESCENS*). Ann. (n. s.) Bot. Gard., Missouri 9: 333-402, illus. 1922.

<sup>7</sup> REDDY, C. S., and GODKIN, J. A BACTERIAL DISEASE OF BROME-GRASS. Phytopathology 13: 75-86, illus. 1923.

usually is somewhat greened, but only slightly as compared with the bright green produced by *Bacterium coronafaciens* var. *atropurpureum*. On Thaxter's potato agar, *Bact. coronafaciens* showed less wrinkling of the surface than the stripe-blight organism. (Fig. 3, A, B.) In beef-peptone bouillon, tubes at first become clouded at the top and bottom with a space between which is only slightly clouded. In three-day cultures, clouding at the bottom of the tube is moderate and in layers and extends about one-third of the distance to the top. Growth in the upper part of the medium is mostly at the surface in the form of a ring or thin pellicle which falls when disturbed. Clouding becomes more uniform as the age of the cultures increases.

In gelatin stab cultures of the stripe-blight organism, growth is best at the top. Liquefaction begins in 2 days and is completed in about 18 days at 20°–22° C. Liquefaction is crateriform, later becoming more infundibuliform to saccate. Cultures of *Bacterium coronafaciens* are only three-fourths liquefied in about 18 days.

#### PHYSIOLOGY

The optimum temperature for growth of both organisms is about 22° C.; the maximum temperature, between 33° and 35° C. Both grew at 1° C.

The thermal death point is about 48° C. Throughout the tests the stripe organism grew after exposure to slightly higher temperatures than *Bacterium coronafaciens*, but only about 1 degree higher.

The optimum reaction for both organisms is between  $P_H$  6.5 and  $P_H$  7.0. Both grew at  $P_H$  5.5 but not at  $P_H$  5.0, and both grew at  $P_H$  8.9 but not at  $P_H$  9.3.

Both organisms grown in Dunham's solution and tested by Ehrlich's method<sup>8</sup> gave negative reactions for indol. A culture of *Bacillus coli*, grown and tested at the same time, gave a positive reaction.

Seven strains, three of halo blight and four of stripe blight, were grown on lead-acetate agar with a reaction of  $P_H$  7.0. All showed a distinct browning of the surface growth and a decided browning of the upper part of the agar as compared with the controls. A small quantity  $H_2S$  was produced by both organisms.

Both organisms are aerobes. Growth occurs only at the surface in stab cultures and in shake cultures of beef-peptone agar plus 1 per cent of glucose, lactose, and mannit, respectively.

In milk cultures both organisms occasionally clear without formation of curd. Usually, however, a soft curd forms in three to five days, followed by slow peptonization, which is completed in from one to two months.

Litmus milk begins to turn blue in two to three days. Reduction begins in about one week and is completed in three to seven days. The blue color gradually returns, first as a light grayish blue, which later becomes dark grayish blue.

Reduction of methylene blue begins in 24 to 48 hours and is completed in about 24 hours. The color returns slowly as a greenish blue. Several tests of cultures of both halo-blight and stripe-blight organisms grown in nitrate bouillon were made with starch water,

<sup>8</sup> Ehrlich's test.—One cubic centimeter of a 2 per cent solution of paradimethyl aminobenzaldehyde in 95 per cent alcohol is added to the culture. Then drop by drop, not more than 0.5 c. c. of concentrated hydrochloric acid is added until a red zone appears between the alcohol and peptone solution.

potassium iodide, and sulphuric acid. The results were negative with all oat cultures, while cultures of *Bacillus coli* gave positive reaction for nitrites. Cultures then were tested on the first, second, fourth, eighth, and tenth days with sulphanilic acid and *a*-naphthylamine. Tests on the eighth and tenth days both gave positive results, showing that with this test both the stripe-blight organism and *Bacterium coronafaciens* should be recorded as nitrate reducers.

Streaks of both organisms on starch agar plates tested with iodine after 12 days showed a clear zone for a centimeter from the margin of the colony.

Several tests were made to detect if possible any difference in acid or alkali production of the two organisms.

Tests for production of ammonia were made in the following ways:

Flasks containing seven-day broth cultures were heated and the steam passed into tubes of Nessler's solution. A yellowish brown precipitate was formed from both cultures and uninoculated controls, but there was much less precipitate from the controls.

Slant cultures then were made on the following medium: Peptone, 40 grams; glucose, 2 grams; dipotassium phosphate, 5 grams; agar, 15 grams; water, 1,000 cubic centimeters.

Tested with 1 per cent phenol and sodium hypochlorite, respectively, the cultures gave negative results, but with Nessler's solution a deep-orange precipitate formed in the inoculated tubes and none in the controls.

The following tests then were made to determine any differences in quantities of ammonia produced by the halo-blight and the stripe-blight organisms.

Cultures were grown in flasks containing beef-peptone broth, asparagin + dextrose in tap water, asparagin + dextrose in distilled water, 1 per cent asparagin in tap water, oat juice diluted, and oatmeal broth. Cultures of different ages were tested for the number of milligrams of nitrogen per liter by distilling off the ammonia into N/10 sodium hydroxide, methyl orange being used as indicator. No significant differences were found.

Tests for the production of acid were made with saccharose, dextrose, levulose, maltose, lactose, glycerin, and mannit in 2 per cent solutions plus 1 and 2 per cents of Difco peptone and Witte's peptone. In all tests there was a strong acid production from saccharose and dextrose and less acid from levulose. The reaction from the other compounds was always alkaline.

Tests then were made to determine whether there were differences in the quantities of acid produced by the two organisms from saccharose and dextrose. In one test with 2 per cent saccharose and 2 per cent dextrose plus 0.2 per cent Difco peptone in distilled water, brom cresol purple and brom phenol blue were added as indicators. In the dextrose cultures containing brom cresol purple, two of the five strains showed a higher acidity than the others, but one was a halo-blight organism and the other a stripe-blight organism. With saccharose the two halo-blight organisms were slightly more acid than the stripe-blight organisms. The acid range of brom phenol blue is too high to show the acid production from saccharose of the

oat organisms, but from dextrose the acid production was about the same for both organisms.

Tests with maltose and lactose in 1 per cent peptone solutions showed alkaline reactions from both organisms.

TABLE 3.—*Acid production, from different sugars in peptone solutions, by stripe-blight and halo-blight organisms, determined by the colorimetric method*

Date inoculated and medium used	Incubation period (days)	PR concentration of medium													Uninoculated
		After inoculation with stripe-blight organism isolation No.—							After inoculation with halo-blight organism isolation No.—						
		66b	69	70	9	10	11	12	Stock	57a	74	75	76		
June 6, 1923.		1	6.2	6.0	6.1	—	—	—	—	6.2	—	—	—	—	6.0
Saccharose (2 per cent) peptone (1 per cent)-----		2	5.8	5.8	6.1	—	—	—	—	6.3	6.2	—	—	—	6.1
		3	5.8	5.7	6.0	—	—	—	—	6.2	6.2	—	—	—	—
		5	5.5	5.8	5.9	—	—	—	—	6.0	6.0	—	—	—	6.1
		9	5.4	5.4	5.7	—	—	—	—	5.8	5.2	—	—	—	6.0
		26	5.2	—	5.3	—	—	—	—	5.2	5.3	—	—	—	—
Nov. 6, 1924:		2	—	—	—	6.0	6.0	5.7	—	—	5.2	5.6	—	5.8	
Dextrose (2 per cent), Difco peptone (0.2 per cent)-----		4	—	—	—	6.2	6.2	5.1	—	—	4.8	5.5	—	5.8	
		7	—	—	—	6.2	6.0	4.4	—	—	4.2	5.3	—	—	
		9	—	—	—	6.1	6.0	4.4	—	—	4.2	5.4	—	5.6	
		11	—	—	—	5.7	6.0	4.4	—	—	4.1	5.0	—	5.7	
		14	—	—	—	5.3	5.8	4.1	—	—	4.1	5.0	—	—	
		18	—	—	—	5.1	5.6	4.1	—	—	4.1	4.5	—	5.7	
		22	—	—	—	5.1	5.4	4.1	—	—	4.0	4.4	—	5.6	
Nov. 6, 1924:		2	—	—	—	6.1	6.2	6.2	—	—	5.7	6.1	—	6.1	
Dextrose (2 per cent), Difco peptone (0.2 per cent), tartrate (0.5 per cent)-----		4	—	—	—	6.1	6.3	5.7	—	—	5.7	6.1	—	6.1	
		7	—	—	—	5.9	6.1	5.6	—	—	5.6	5.9	—	—	
		9	—	—	—	6.0	5.9	5.6	—	—	5.5	5.8	—	6.1	
		11	—	—	—	5.7	6.0	5.4	—	—	5.5	5.8	—	6.1	
		14	—	—	—	5.6	5.6	5.5	—	—	5.4	5.8	—	—	
		18	—	—	—	5.7	5.5	5.7	—	—	5.4	5.7	—	6.1	
		22	—	—	—	5.0	5.5	5.5	—	—	5.4	5.6	—	6.1	
Nov. 26, 1924:		2	—	—	—	5.8	5.8	5.8	5.9	—	5.4	6.1	5.8	5.9	
Saccharose (2 per cent), Difco peptone (0.2 per cent), distilled water-----		3	—	—	—	5.7	5.6	5.6	5.8	—	4.9	5.8	5.7	5.8	
		5	—	—	—	5.7	5.6	5.7	5.8	—	4.2	5.9	5.9	5.8	
		8	—	—	—	5.9	5.8	5.6	5.8	—	4.2	5.8	5.9	—	
		11	—	—	—	5.8	5.8	5.6	5.5	—	3.8	5.7	5.4	—	
		14	—	—	—	5.5	5.6	5.0	4.7	—	3.6	5.5	5.7	5.8	
		17	—	—	—	5.3	5.4	4.8	4.5	—	3.7	5.0	4.6	—	
		22	—	—	—	4.2	5.1	4.1	4.3	—	3.8	5.2	4.2	5.9	
		32	—	—	—	4.1	4.8	4.0	4.3	—	3.8	5.4	4.0	6.2	
	Nov. 26, 1924.		2	—	—	—	5.5	5.7	5.4	5.5	—	4.8	5.4	5.6	5.6
Dextrose (2 per cent), Difco peptone (0.2 per cent), distilled water-----		3	—	—	—	5.4	5.7	5.4	5.4	—	4.5	5.4	5.5	5.6	
		5	—	—	—	5.4	6.0	5.1	5.5	—	4.1	5.4	5.4	5.6	
		8	—	—	—	5.3	6.1	5.0	5.1	—	4.0	5.4	5.4	—	
		11	—	—	—	4.1	5.6	4.4	4.2	—	3.6	5.0	4.8	—	
		14	—	—	—	4.1	5.1	3.9	4.5	—	3.7	4.6	4.3	5.5	
		17	—	—	—	4.1	4.6	4.2	3.9	—	3.6	4.6	3.9	—	
		22	—	—	—	4.0	4.2	3.8	4.3	—	3.6	4.3	4.1	5.4	
		32	—	—	—	4.0	4.0	3.8	4.2	—	3.6	4.2	4.2	5.7	
	Nov. 18, 1924:		2	—	—	—	5.7	5.7	6.0	5.8	—	5.5	5.8	5.8	5.6
Dextrose (2 per cent), Difco peptone (0.2 per cent), in Altman at 18° C-----		4	—	—	—	5.8	5.7	6.0	5.8	—	5.6	5.8	5.8	—	
		6	—	—	—	5.9	6.0	6.4	5.8	—	5.5	5.9	5.9	—	
		8	—	—	—	5.8	5.9	6.4	5.9	—	5.2	5.9	5.9	—	
		12	—	—	—	5.8	6.1	6.4	5.9	—	5.3	5.9	5.9	5.8	
		16	—	—	—	5.7	5.9	6.4	5.9	—	4.8	5.9	5.9	—	
	Jan. 3, 1925:		2	—	—	—	5.8	6.0	6.0	5.8	—	5.7	5.9	5.6	5.8
Dextrose (2 per cent), Difco peptone (0.2 per cent)-----		3	—	—	—	5.8	6.1	6.2	5.9	—	5.5	5.9	5.0	—	
		4	—	—	—	5.9	6.3	6.2	5.9	—	5.1	5.9	5.8	5.6	
		6	—	—	—	6.4	6.5	6.3	5.9	—	5.2	5.8	5.0	—	
		7	—	—	—	6.4	6.0	6.4	5.9	—	4.8	5.8	4.7	5.8	

TABLE 3.—*Acid production, from different sugars in peptone solutions, by stripe-blight and halo-blight organisms, determined by the colorimetric method—Contd.*

Date inoculated and medium used	Incuba- tion period (days)	P <sub>H</sub> concentration of medium												Unin- ocu- lated
		After inoculation with stripe- blight organism isolation No.—						After inoculation with halo-blight organism isolation No.—						
		66b	69	70	9	10	11	12	Stock	57a	74	75	76	
Jan. 12, 1925.														
Dextrose (2 per cent), Difco peptone (0.2 per cent) -----	1 {a.m.				6.1	6.1	6.1	6.2			5.7	6.0	6.2	5.9
	1 {p.m.				5.8	5.9	6.0	5.9			5.8	5.9	5.9	
	2 {a.m.				5.8	6.0	6.1	5.9			5.1	5.7	5.9	5.7
	2 {p.m.				5.8	6.0	6.1	5.9			5.1	5.8	5.9	
	3 {a.m.				5.9	6.0	6.2	5.8			5.2	5.7	5.6	5.7
	3 {p.m.				5.9	6.1	6.1	5.8			4.8	5.4	5.6	
	4 {a.m.				6.0	5.8	6.0	5.8			4.2	5.4	5.3	5.7
	4 {p.m.				5.9	5.9	6.0	5.8			4.3	5.1	5.7	
	5 a.m.				5.9	5.9	6.3	5.8			4.5	5.2	5.9	5.7
	7 {a.m.				6.2	5.9	5.9	5.8			4.1	4.9	4.8	
	7 {p.m.				5.9	6.0	5.9	5.8			4.2	4.9	5.4	5.7
	8 a.m.				6.0	5.9	5.8	5.8			4.1	5.0	6.2	
9 a.m.				5.9	5.8	5.8	5.8			3.9	4.7	4.6	7.1	
10 p.m.				6.2	5.8	5.6	5.8			4.0	4.8	4.5		
11 p.m.				5.9	5.6	6.2	5.8			4.2	5.4	4.3		
12 p.m.				6.4	5.8	5.8	5.9			4.0	5.4	4.5		
Feb. 2, 1925:														
Synthetic medium -----	1				6.9	7.0	6.9	6.9			6.9	7.0	6.9	7.0
	2				6.8	6.9	6.9	6.9			6.8	6.8	6.8	7.1
	3				6.8	6.8	6.7	6.7			6.7	6.7	6.7	
	4				6.7	6.7	6.6	6.7			6.6	6.5	6.6	
7				6.4	6.4	6.2	5.9			6.2	6.2	6.6		
Feb. 9, 1925:														
	1				6.8	6.8	6.8	6.8			6.7	6.8	6.8	6.9
	2				6.7	6.8	6.8	6.8			6.7	6.7	6.7	6.9
	5				6.6	6.6	6.6	6.6			6.6	6.5	6.6	
	7				6.5	6.6	6.6	6.6			6.6	6.6	6.5	
	8				6.4	6.5	6.5	6.5			6.5	6.5	6.4	
	9				6.2	6.3	6.5	6.4			6.4	6.5	6.4	
	10				6.3	6.3	6.4	6.4			6.2	6.5	6.5	
	12				6.2	6.2	6.3	6.5			5.9	6.4	6.3	
	15				5.8	5.9	5.8	6.0			5.9	6.3	6.2	
	16				5.9	6.0	5.9	6.4			5.8	5.7	6.0	
	18				5.8	6.0	5.8	6.0			5.7	6.1	5.8	
	19				5.7	5.8	5.7	6.1			6.1	5.6	6.1	
	22				5.5	5.4	5.9	5.7			5.5	5.5	5.6	
	25				5.7	5.7	5.9	5.7			5.7	5.6	5.5	
	33				5.2	5.2	5.5	5.7			5.6	5.4	5.2	

\* Synthetic medium, Manual of the Society of American Bacteriologists: Monobasic ammonium phosphate, 1 gm.; potassium chloride, 0.02 gm.; magnesium sulphate, 0.02 gm.; dextrose, 10 gm.; water, 1,000 c. c.

Table 3 gives the results of tests for acid production from saccharose, dextrose, and synthetic media, using the colorimetric method.

While in some instances the halo-blight organisms produced more acid than the stripe-blight organisms, the results as a whole show no consistent differences in quantities of acid produced.

#### TECHNICAL DESCRIPTION

##### **Bacterium striafaciens n. sp.**

A motile rod with rounded ends and polar flagella; single, in pairs, or in short chains; average measurements  $0.66 \times 1.76 \mu$ ; no spores; capsules formed; aerobic; white on agar; liquefies gelatin; does not produce indol; produces  $H_2S$ ; forms a soft curd in milk, which is slowly peptonized; slight reduction of nitrates; starch hydrolyzed; ammonia produced; acid produced from dextrose, saccharose, and levulose, none from other carbon compounds tested; optimum temperature  $22^\circ C$ ., thermal death point  $48^\circ$ ; optimum reaction  $P_H$  6.5 to  $P_H$  7.0; Gram-negative; not acid fast; pathogenic on varieties of cultivated oats and to a slight degree on barley.

## SUMMARY

A new bacterial disease of oats has been collected from the chief oat-growing sections of the United States. In the fields of oats observed by the writer the disease was not sufficiently abundant to make control measures necessary. Specimens from the Pacific coast, however, seem to be more heavily infected.

The lesions are water-soaked dots and streaks which later turn rusty brown. They show no halolike borders and under favorable conditions are covered with shining dried scales of exudate.

These stripe lesions are distinctly different from the lesions produced by *Bacterium coronafaciens* Elliott, *Bact. coronafaciens* var. *atropurpureum* Reddy and Godkin, and *Pseudomonas alboprecipitans* Rosen.

Repeated isolations have given a typical white organism which on inoculation produced typical stripe lesions quite different from the lesions of the halo-blight organism.

Inoculations of a large number of oat varieties have shown the same differences in varietal susceptibility to both stripe-blight and halo-blight organisms. The host range of the two organisms also was the same as far as tested.

No consistent differences in the reactions of the two organisms to various culture media have been detected.

Morphologically the stripe-blight organism is consistently smaller than *Bacterium coronafaciens*.

On the basis of the differences in type of lesions produced on oats and the smaller size of the stripe-blight organisms, the name *Bacterium striafaciens* n. sp. is suggested for the organism producing water-soaked dots and streaks on oats.

# A COMPARATIVE STUDY OF THE QUINHYDRONE AND HYDROGEN ELECTRODES FOR DETERMINING THE HYDROGEN-ION CONCENTRATION OF SOILS<sup>1</sup>

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## INTRODUCTION

The determination of the hydrogen-ion exponent of soils heretofore has been confined to two methods, the hydrogen electrode and the colorimetric. With the development of the quinhydrone electrode for practical use and its adaptation to soils by Biilmann (3)<sup>2</sup>, extensive application of this electrode procedure has been made by virtue of its comparative simplicity and economy of time. In addition, when the presence of nitrates is sufficiently high to act as an interfering factor in the obtainment of constant potentials as a result of reduction by the hydrogen electrode, the quinhydrone electrode, as shown by Biilmann, may be used without danger of error from this source.

Results of hydrogen-ion exponent determinations on soils by the hydrogen and quinhydrone electrodes indicate excellent agreement as reported by some soil investigators. However, results to the contrary have also been reported. Christensen and Jensen (8) determined the  $P_H$  values of suspensions of many diverse Danish soils, ranging in  $P_H$  value from approximately 3.5 to 8.5, and found close agreement between the hydrogen and quinhydrone electrodes. Baver (2) reports that the hydrogen and quinhydrone electrode results compare closely, the variation ranging from 0 to 0.2  $P_H$ . When working on soil suspensions Brioux and Pien (7), however, were unable to obtain in general the agreement between the two electrodes as reported by Christensen and Jensen. When centrifugates were used the agreement was better, although the quinhydrone results were slightly higher as a rule and there still remained cases of unexplainable deviations.

The present work was therefore undertaken to compare the two electrodes as used for the determination of the hydrogen-ion exponents of soil suspensions.

## THEORETICAL CONSIDERATIONS

The familiar working formula for the computation of  $P_H$  values, using the hydrogen electrode is:

$$(1) \quad P_H = \frac{E - E_{(cal.)}}{0.00019837 T}$$

where—

$E$  = observed potential.

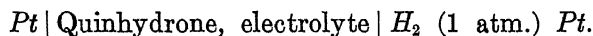
$E_{(cal.)}$  = potential between calomel electrode and normal hydrogen electrode at  $t$  centigrade.

$T$  = absolute temperature ( $273.09 + t$  centigrade).

<sup>1</sup> Received for publication Aug. 15, 1927; issued December, 1927.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 834.

Billmann and his coworkers (4, 5, 6) determined the potential difference of cells of the type



where the quinhydrone and hydrogen electrodes were in the same electrolytes of known  $P_H$  value, contact potential being eliminated by connecting the two electrodes with the common electrolyte. The measurements were made at 0°, 18°, 25°, and 37° C., respectively, and it was found that within the limits of experimental accuracy, the potential-temperature curve of the quinhydrone electrode over this range is practically a straight line represented by the following equation:

$$(2) \quad \pi_t = 0.7175 - 0.00074 t$$

where the potential of the electrode at  $t^\circ$  and 760 mm. dry hydrogen is represented by  $\pi_t$ , the quinhydrone electrode being positive to the hydrogen electrode. The largest difference between the calculated and observed potentials was found to be 0.4 millivolts. By a simple substitution in which  $E$  in equation (1) is replaced by  $\pi_t - E_q$  where  $E_q$  is the observed potential difference between the quinhydrone and calomel electrodes, the working equation for the quinhydrone electrode becomes:

$$(3) \quad P_H = \frac{\pi_t - E_{(cal.)} - E_q}{0.00019837 T}$$

The standard calomel electrode may be replaced by a standard quinhydrone electrode as suggested by Veibel (16). However, in the present work the saturated calomel electrode was used by preference and assigned the  $E_{(cal.)}$  values given by Clark (9).

The quinhydrone electrode results are calculated from equation (3), where the  $\pi_t$  and  $E_{(cal.)}$  values are referred to a hydrogen partial pressure of 760 mm.

When employing either the saturated, normal, or tenth-normal calomel electrodes there is a current reversal at a definite  $P_H$  value depending upon the electrode used and the temperature. From equation (3) it is obvious that with the tenth-normal calomel electrode at 18° C. the potential becomes zero at about  $P_H$  6.34 and negative with increasing  $P_H$ . With the saturated calomel electrode at 18° this change of potential from positive to negative occurs at about  $P_H$  7.86. With those soils below  $P_H$  7.86 the observed potentials when using the saturated calomel electrode will be positive, since the quinhydrone electrode will be positive to the calomel electrode, and above  $P_H$  7.86 the potentials will be negative.

The sign of the observed potential difference is that of the quinhydrone electrode in relation to the known sign of the standard calomel electrode.

## EXPERIMENTAL WORK

### QUINHYDRONE CONCENTRATION

After a few preliminary experiments in which the quinhydrone concentration was varied from about 0.02 gm. to approximately 0.5 gm., using the soil-water ratio of 1 to 2, it was concluded that about 0.1 gm. of quinhydrone was sufficient for soil suspensions.

Three grades of quinhydrone were used, one a high-grade purchased product m. p.  $169^{\circ}$ – $170^{\circ}$  and the remaining two prepared in the laboratory by the method of Biilmann and Lund (6). Results with the three preparations were in agreement.

Introduction of the quinhydrone to the soil suspension was facilitated by means of a glass spoon constructed so as to deliver about 100 mgm. of quinhydrone.

Baver (2) used in his work on the quinhydrone electrode 0.05 gm. of quinhydrone per 15 c. c. of solution, and Hissink and Van der Spek (13) 0.05 gm. for 5 gm. of soil and 20 c. c. of water.

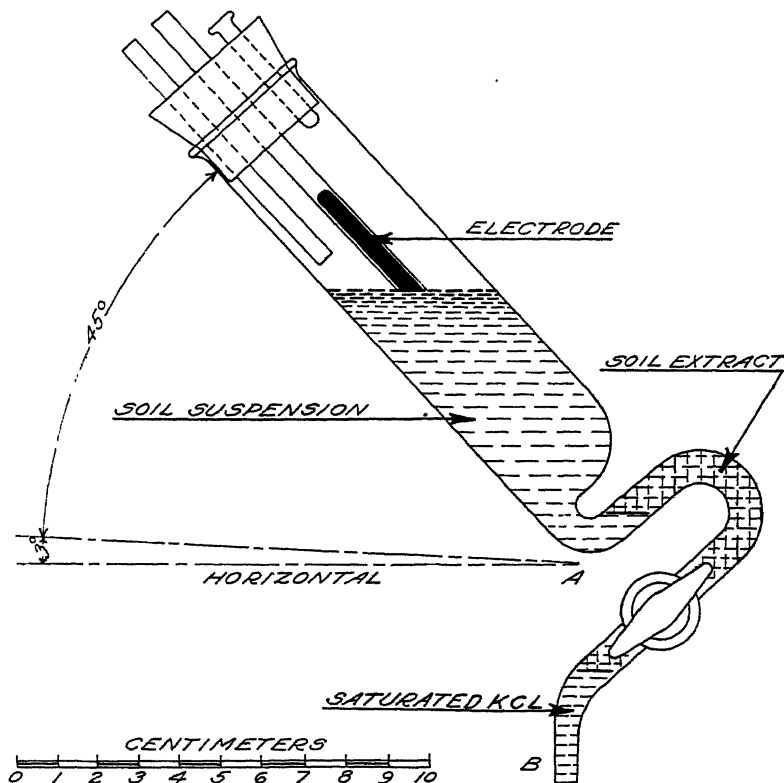


FIG. 1.—The electrode vessel

#### SOIL-WATER RATIO

All measurements, electrometric and colorimetric, were made on soil suspensions with a soil-water ratio of 1 to 2, using 15 gm. of air-dried soil (passing a 2 mm. sieve) and 30 c. c. of water. This mixture was shaken by hand in a large pyrex test tube for approximately 1 minute and allowed to stand about 20 minutes for sedimentation.

The colorimetric measurements were made on the centrifugate of the supernatant liquid, employing Gillespie's drop-ratio method (11), with the substitution of bromocresol green (14) for methyl red.

#### ELECTRODE VESSEL

The electrode vessel of pyrex glass shown in Figure 1 is a closed type, being a modification of the one employed by Gillespie (10),

and was used for both quinhydrone and hydrogen electrode measurements. The volume of the vessel is about 65 c. c., the ratio of the volume to that of the soil suspension being such as to insure thorough mixing during shaking.

The application to soils of the principle of shaking the electrode vessel with alternate exposure and immersion of the electrode, especially in hydrogen electrode measurements, allows of a more rapid attainment of equilibrium between gaseous hydrogen, the electrode, and the soil suspension. A localized equilibrium with this procedure is avoided, and one is obtained which is more characteristic of the entire suspension.

#### ELECTRODE

The electrode shown in Figure 2 consists of a sheet of platinum foil about 2.5 by 3.0 cm. securely held in place by two platinum leads for the current, which were welded to both ends of the foil and sealed

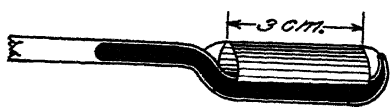


FIG. 2.—The electrode

into the glass support. The electrodes, while occasionally heated to redness in the alcoholic flame, as suggested by Billmann (3) for the quinhydrone electrode procedure, were also frequently treated with hot chromic-acid solution and when not in use were kept in a chromic-acid bath. The gold-foil electrodes were about 1.0 by 2.0 cm. and were welded to a short piece of platinum wire and then sealed into a glass tube.

#### VARIATION OF QUINHYDRONE ELECTRODE MEASUREMENTS WITH TIME

To determine the variation of the quinhydrone electrode with time, different soil types were investigated. Potential measurements were made on suspensions of duplicate samples of these soils immediately after an initial shaking of the electrode vessel and then after definite intervals with the vessel at rest. The potential differences given in Table 1 are characteristic of several duplicate measurements made on each sample and are typical of a large number of results.

A large number of potentials measured immediately after an initial shaking of about 30 seconds and then after the following 1, 5, and 10 minute intervals, with the vessel at rest, were similar to those shown in Table 1.

In Table 1 the initial time of shaking the electrode vessel has been increased to 2 minutes, and the potentials read immediately after shaking and after the following intervals indicated. In those instances where there was a final shaking of the electrode vessel for 2 minutes, little further change in the potentials was observed. The constancy of potential of the acid soils may in general be considered good over the 15-minute period, although there was a small potential decrease or corresponding  $P_H$  increase with time.

With the alkaline soils the potentials maintained as a rule a good degree of constancy over the 15-minute period, in some instances as high as about  $P_H$  9.0. There were, however, occasional exceptions to this on both the acid and alkaline sides. Quinhydrone electrode measurements on one black alkali soil of  $P_H$  9.0 (hydrogen electrode value) drifted rapidly over a 15-minute period, the initial quinhydrone

value being about  $P_H$  9.5. In instances of this nature the drift was such that any measurement is admittedly a "forced" one.

TABLE 1.—*Variation of quinhydrone electrode measurements with time*

Soil type	Electrode vessel shaken 2 minutes		Electrode vessel at rest						Electrode vessel shaken 2 minutes	
			5 minutes		10 minutes		15 minutes			
	Volts	$P_H$	Volts	$P_H$	Volts	$P_H$	Volts	$P_H$	Volts	$P_H$
Norfolk fine sandy loam, Norfolk, Va.	0.1729	4.73	0.1712	4.76	0.1704	4.77	0.1699	4.78	0.1683	4.79
	.1734	4.72	.1722	4.74	.1711	4.76	.1707	4.77	.1700	4.78
Caribou loam, Aroostook County, Me.	.1706	4.82	.1678	4.87	.1668	4.88	.1658	4.90	.1651	4.91
	.1679	4.86	.1658	4.90	.1644	4.92	.1643	4.93	.1640	4.93
Washburn loam, Aroostook County, Me.	.1230	5.47	.1209	5.50	.1184	5.54	.1180	5.55	.1176	5.56
	.1214	5.50	.1195	5.54	.1187	5.55	.1174	5.57	.1176	5.57
Portsmouth fine sandy loam, New Bern, N. C.	.1196	5.60	.1194	5.60	.1187	5.62	.1185	5.62	.1182	5.62
	.1198	5.60	.1189	5.61	.1175	5.64	.1173	5.64	.1170	5.64
Miami silty clay loam, Wells County, Ind.	.0151	7.40	.0160	7.39	.0160	7.39	.0169	7.37	-----	-----
	.0128	7.44	.0126	7.44	.0128	7.44	.0139	7.42	-----	-----
Stockton clay adobe, Butte County, Calif.	-.0456	8.41	-.0522	8.52	-.0542	8.56	-.0547	8.57	-.0548	8.57
	-.0448	8.40	-.0501	8.49	-.0560	8.59	-.0562	8.59	-.0547	8.57
Prowers clay loam, Lamar, Colo.	-.0434	8.36	-.0431	8.35	-.0428	8.35	-.0425	8.34	-.0405	8.31
	-.0425	8.34	-.0431	8.35	-.0433	8.36	-.0430	8.35	-.0411	8.32
Fort Lyon clay loam, Las Animas, Colo.	-.0347	8.26	-.0390	8.33	-.0390	8.33	-.0386	8.32	-.0370	8.30
	-.0374	8.30	-.0387	8.33	-.0386	8.32	-.0386	8.32	-.0364	8.29
Minnequa silty clay loam, Rocky Ford, Colo.	-.0646	8.73	-.0671	8.77	-.0673	8.78	-.0666	8.77	-.0631	8.71
	-.0639	8.72	-.0651	8.74	-.0656	8.75	-.0656	8.75	-.0620	8.69
Fallon loam, Churchill County, Nev.	-.0852	9.20	-.0905	9.29	-.0905	9.29	-.0930	9.33	-.0910	9.30
	-.0860	9.21	-.0878	9.24	-.0893	9.27	-.0897	9.28	-.0877	9.24

Quinhydrone electrode measurements were also made at intervals over a 10-minute period, with the electrode vessel shaking continuously as in the procedure recommended by Gillespie (10) for hydrogen electrode determinations. The potentials were in general similar to those recorded in Table 1, in which instance the electrode vessel was not shaken continuously.

As a result of many observations it seems advisable to make the potential measurement about one-half to one minute after the addition of the quinhydrone.

#### QUINHYDRONE ELECTRODE PROCEDURE

The procedure adopted for the determination of the  $P_H$  values of soils with the quinhydrone electrode is as follows: Fifteen grams of air-dried soil and 30 c. c. of water are shaken in a large pyrex test tube approximately one minute. This suspension is allowed to stand a short time for sedimentation, and a portion of the supernatant liquid sufficient to fill the neck of the vessel and bore of the stopcock is poured into the electrode vessel. (Fig. 1.) The end *B* is filled with saturated potassium-chloride solution and the vessel attached to a shaker, the actuating arm of which passes through the axis *A*. The end *B* is then connected to the saturated calomel electrode. The remainder of the soil suspension is poured into the vessel and about 0.1 gm. of quinhydrone added. The plain platinum electrode (fig. 2) is placed in the electrode vessel, which is shaken one-half to one minute. The potential is then read immediately with the vessel at rest.

Contact with the calomel electrode is drawn into the end *B*, thus preventing the entrance of potassium chloride into the soil suspension. The potential is measured with a Leeds & Northrup type K potentiometer in connection with a high-grade galvanometer and a Bureau

of Standards certified Weston cell. The recorded temperature, which was that of a small room especially adapted to potentiometric work, did not vary greatly during the day; however, temperature corrections were applied to all measurements.

#### HYDROGEN ELECTRODE PROCEDURE

The procedure followed for the hydrogen electrode determination is as follows: The soil treatment and filling of the electrode vessel are as described for the quinhydrone electrode procedure prior to adding the quinhydrone. The electrode (fig. 2) was lightly coated with palladium in about 5 seconds, electrolyzed in dilute sulphuric acid, thoroughly washed in distilled water, then placed in the vessel (fig. 1), and the space above the soil suspension thoroughly swept out with pure hydrogen, but not bubbled through the suspension. During shaking, which is at the rate of about 80 complete swings per minute, contact with the calomel electrode is drawn into the end *B* and the potential read after 5-minute intervals, the second reading being a control. Equilibrium is generally obtained in 5 to 10 minutes.

#### VARIATION OF HYDROGEN ELECTRODE MEASUREMENTS WITH TIME

The hydrogen electrode measurements shown in Table 2 were made after the indicated periods, during and after shaking of the electrode vessel.

TABLE 2.—*Variation of hydrogen electrode measurements with time*

Soil type	Electrode vessel shaking						Electrode vessel at rest							
	5 minutes		10 minutes		15 minutes		1 minute		5 minutes		10 minutes		15 minutes	
	Volts	$P_H$	Volts	$P_H$	Volts	$P_H$	Volts	$P_H$	Volts	$P_H$	Volts	$P_H$	Volts	$P_H$
Norfolk fine sandy loam,	0.5279	4.75	0.5296	4.77	0.5293	4.78	0.5298	4.79	0.5298	4.79	0.5298	4.79	0.5298	4.79
Norfolk, Va.	.5283	4.76	.5283	4.76	.5286	4.77	.5290	4.77	.5291	4.77	.5291	4.77	.5291	4.77
Caribou loam, Aroostook	.5296	4.75	.5311	4.77	.5298	4.75	.5323	4.79	.5330	4.80	.5328	4.80	.5329	4.80
County, Me.	.5307	4.79	.5312	4.79	.5307	4.79	.5335	4.83	.5342	4.84	.5346	4.85	.5351	4.86
Washburn loam, Aroostook	.5709	5.45	.5699	5.43	.5699	5.43	.5721	5.47	.5728	5.48	.5737	5.49	.5741	5.50
County, Me.	.5715	5.46	.5702	5.44	.5694	5.42	.5725	5.47	.5736	5.49	.5743	5.50	.5746	5.51
Portsmouth fine sandy	.5844	5.68	.5831	5.66	.5830	5.66	.5835	5.66	.5832	5.66	.5832	5.66	.5832	5.66
loam, New Bern, N. C.	.5823	5.64	.5814	5.63	.5818	5.64	.5818	5.64	.5818	5.64	.5818	5.64	.5818	5.64
Miami silty clay loam,	.6902	7.49	.6877	7.45	.6858	7.42	.6903	7.49	.6899	7.49	.6894	7.48	.6882	7.46
Wells County, Ind.	.6873	7.44	.6870	7.44	.6861	7.42	.6884	7.46	.6883	7.46	.6883	7.46	.6883	7.46
Stockton clay adobe, Butte	.7137	7.91	.7145	7.93	.7138	7.91	.7170	7.97	.7174	7.99	.7161	7.95	.7160	7.95
County, Calif.	.7124	7.89	.7116	7.88	.7116	7.88	.7146	7.93	.7154	7.94	.7146	7.93	.7147	7.93
Prowers clay loam, Lamar,	.7186	7.97	.7189	7.98	.7185	7.97	.7195	7.99	.7197	7.99	.7198	7.99	.7200	7.99
Colo.	.7185	7.97	.7183	7.97	.7178	7.96	.7194	7.98	.7196	7.99	.7196	7.99	.7197	7.99
Fort Lyon clay loam, Las	.7200	8.01	.7187	7.99	.7183	7.98	.7210	8.03	.7209	8.03	.7207	8.02	.7207	8.02
Animas, Colo.	.7200	8.01	.7183	7.98	.7177	7.97	.7203	8.02	.7204	8.02	.7204	8.02	.7203	8.02
Minnequa silty clay loam,	.7509	8.52	.7496	8.50	.7489	8.49	.7502	8.51	.7504	8.52	.7509	8.52	.7509	8.52
Rocky Ford, Colo.	.7482	8.48	.7478	8.47	.7475	8.47	.7503	8.51	.7510	8.53	.7513	8.53	.7513	8.53
Fallon loam, Churchill	.7911	9.17	.7912	9.17	.7917	9.18	.7949	9.22	.7944	9.23	.7941	9.22	.7938	9.22
County, Nev.	.7912	9.17	.7891	9.14	.7890	9.14	.7929	9.20	.7930	9.20	.7918	9.18	.7920	9.19

The results indicate in general the reproducibility and constancy that may be obtained, and are quite representative of several thousand determinations made during the last eight years, being exclusive, however, of those occasional instances of erratic potentials referred to below.

Gillespie and Hurst (12) have given a frequency table showing the number of times potential changes were observed due to 5 minutes' extra shaking. The readings were made during shaking after 5-

minute intervals, the second reading being a control. As a result of 58 determinations the mean potential drop due to the extra 5 minutes' shaking was 0.9 millivolt. It is stated by these authors that "if the electrode vessel is not shaken during the measurement the potential is not constant, but becomes numerically greater, sometimes so fast that any measurement would be a forced one." This, they say, is due to the presence of nitrates and possibly to other oxidizing agents. With palladium black electrodes a similar potential rise after bringing the electrode vessel to rest has been observed, particularly with alkaline soils when using impure hydrogen containing small quantities of oxygen. Under similar conditions, electrodes of platinum or iridium black on platinum or gold foil showed insignificant potential variations.

It has been observed, as further stated by Gillespie and Hurst (12), that "if the vessel were not shaken during the measurements, and a final potential reading were taken after the potential seemed to become constant, the results in some cases would be quite erroneous, and it would appear that in general such results would be more or less in error." The results given in Table 2 seem to bear out Gillespie's statement of the constancy of potential during shaking of the electrode vessel.

As a rule the reproducibility of hydrogen electrode measurements on soil suspensions has been within about 3 millivolts. It should be remembered, however, that the hydrogen electrode is not universally applicable, especially in the presence of unsaturated substances and active oxidizing agents, and there may be soils containing constituents which would cause unsatisfactory results and preclude the use of the hydrogen electrode. In instances of this nature, which may occur occasionally, or where the investigator has reason to suspect the validity<sup>3</sup> of his results, or when there is difficulty in obtaining stable and reproducible potentials with the hydrogen electrode, it is advisable to compare the hydrogen electrode, the quinhydrone electrode, and the colorimetric methods with one another.

#### PALLADIUM, PLATINUM, AND IRIIDIUM BLACK ELECTRODES

Experiments with hydrogen electrodes of palladium, platinum, or iridium black on platinum or gold foil showed that, under equilibrium conditions, results on soil suspensions by these different electrodes are in close agreement.

Palladium, platinum, and iridium black were lightly deposited in about 5 seconds from approximately 3 per cent solutions of palladium chloride, chloroplatinic acid, and iridium sesquichloride, respectively, each containing about 2 per cent of hydrochloric acid.

In regard to plating electrodes it might be well to mention at this point that it does not seem improbable that the properties of the plating solution as well as the current density employed are factors contributory to the character of the deposit. As stated by Clark (9), "since there is a simultaneous deposit of metal and hydrogen and,

<sup>3</sup> A soil sample submitted to the writer gave with a hydrogen electrode a negative potential and a correspondingly absurd  $P_H$  value for a soil. It was noticed that the box in which the soil had been sent was labeled "Bichloride of Mercury." The treatment of 15 gm. of a similar sandy soil low in organic matter with as little as 10 mgm. of  $HgCl_2$  produced an effect greater than that mentioned above, this apparently being an instance of electrode poisoning. The effect on other soils higher in organic matter was comparatively small. A second sample of the original soil, but free from  $HgCl_2$ , was found to have a  $P_H$  value of 5.3.

since the character of the platinum, palladium, or iridium black is undoubtedly due to the vigor of the hydrogen evolution, it is evident that the  $P_H$  of the solution constitutes a part of the conditions." In this connection Andrews (1) states that "palladium electrodes for hydrogen-ion determinations are much less reliable than platinum electrodes because of the lack of permanence of the former which results from a more or less rapid change of amorphous to crystalline palladium."

### COMPARISON OF RESULTS

Some of the results characteristic of those obtained by using the two procedures outlined for the quinhydrone and hydrogen electrodes are shown in Tables 3 and 4, together with a few available colorimetric results.

TABLE 3.—Comparison of  $P_H$  results on soils

Hydrogen electrode	Quinhydrone electrode	Hydrogen electrode	Quinhydrone electrode	Hydrogen electrode	Quinhydrone electrode	Colorimetric	Hydrogen electrode	Quinhydrone electrode	Colorimetric
$P_H$	$P_H$	$P_H$	$P_H$	$P_H$	$P_H$	$P_H$	$P_H$	$P_H$	$P_H$
3.92	4.10	5.41	5.48	7.48	7.42	7.5	8.13	8.34	7.9
4.20	4.13	5.47	5.50	7.54	7.87	7.6	8.14	8.16	7.9
4.26	4.40	5.53	5.65	7.60	7.77	7.5	8.20	8.00	8.1
4.52	4.47	5.61	5.76	7.61	7.79	7.6	8.20	8.35	7.9
4.53	4.48	5.62	5.67	7.64	7.85	7.7	8.20	8.49	7.9
4.78	4.75	5.80	5.75	7.72	8.00	7.5	8.21	8.38	8.0
4.81	4.88	6.00	6.05	7.82	8.31	7.7	8.31	8.53	8.1
4.90	4.80	6.00	6.20	8.00	8.20	8.0	8.36	8.37	8.2
4.98	4.98	6.00	6.30	8.00	8.35	8.0	8.51	8.61	8.5
5.00	5.20	6.09	6.00	8.00	8.40	8.0	8.62	8.60	8.5
5.04	5.00	6.20	6.40	8.04	8.34	7.9	8.64	8.79	Turbid.
5.07	4.90	6.64	6.80	8.07	8.24	8.1	8.85	8.96	Turbid.
5.28	5.17	6.80	7.14	8.10	8.30	8.0	9.00	9.50	Turbid.
5.36	5.66	7.46	7.50	8.10	8.30	7.8	9.17	9.20	Turbid.

The agreement between the two methods as shown in Table 3 may, with the exception of a few values around  $P_H$  8.0, be considered good for soils, particularly on the acid side. On the alkaline side the larger variations were more frequent, although instances of good agreement were obtained as high as  $P_H$  9.2. In those instances where the colorimetric results are given, there is in general better agreement with the hydrogen than with the quinhydrone electrode results, the quinhydrone electrode in these instances apparently being in error.

La Mer and Parsons (15) found that the quinhydrone electrode gave reliable results in buffered solutions more acid than  $P_H$  8.0, provided no rapid oxidizing or reducing agents were present. Erroneous results obtained in more alkaline solutions are attributed to the autoxidation of hydroquinone.

In an article by Büllmann (4) there is discussed the influence on the potential of chemical compounds present in the quinhydrone electrode, such as the acidity of the hydroquinone, the dissociation and the influence of acids and salts on the activities of hydroquinone and quinone. It is also stated by this author (and this point should be kept in mind) that "the quinhydrone electrode is by no means a universal electrode, and there may be soils containing constituents which affect the electrode potential."

TABLE 4.—Comparison of  $P_H$  results on buffer solutions

Electrode	$P_H$ values							
Hydrogen.....	1.50	2.99	4.58	7.49	7.98	8.25	8.59	8.94
Quinhydrone.....	1.53	3.02	4.56	7.48	7.96	8.20	8.55	8.90

The hydrogen and quinhydrone electrode results given in Table 4 for some of Clark and Lubs's (9) buffer solutions ranging from about  $P_H$  3.0 to 8.0 are in excellent agreement, the largest deviation between the two electrodes being 0.03  $P_H$ . Above  $P_H$  8.0 the quinhydrone electrode potentials were not so constant but continued to drift over a 15-minute period. From  $P_H$  8.0 to about 9.0, as far as these investigations were carried, readings made prior to any appreciable drift; that is, about 1 minute after the addition of quinhydrone, gave results agreeing closely with those of the hydrogen electrode. At about  $P_H$  9.0 the drift of the quinhydrone electrode potentials over a period of 15 minutes had risen to about 6 millivolts.

Many quinhydrone electrode potentials obtained with platinum and gold-foil electrodes on some of Clark and Lubs's buffer solutions ranging from about  $P_H$  2.3 to 8.0 showed that the potentials obtained with gold-foil electrodes at intervals of  $\frac{1}{2}$ , 1, 5, and 10 minutes after the addition of quinhydrone were more nearly constant than those observed with platinum electrodes. With gold electrodes as a rule a constant oxidation-reduction potential was quickly established, whereas with platinum electrodes there was generally a slight drift toward more negative potentials. The  $P_H$  values obtained with gold quinhydrone electrodes were as a rule in closer agreement with the hydrogen electrode values than those obtained with platinum quinhydrone electrodes.

#### SUMMARY

Many results on soils from about  $P_H$  4.0 to 9.0 indicate that the constancy of the quinhydrone electrode measurements may, with an occasional exception, be considered good.

Hydrogen electrode measurements of acid and alkaline soils from about  $P_H$  4.0 to 9.0 made on soil suspensions, using a soil-water ratio of 1 to 2, were on the whole reproducible and constant. Equilibrium was generally obtained in 5 to 10 minutes.

Results on soil suspensions with hydrogen electrodes of palladium, platinum, or iridium black on platinum or gold foil were under equilibrium conditions in close agreement.

Results on soil suspensions from about  $P_H$  4.0 to 9.0 by the hydrogen and quinhydrone electrode methods showed on the whole good agreement for soils, particularly on the acid side. On the alkaline side the larger variations were more frequent.

There was excellent agreement between results obtained by the quinhydrone and hydrogen electrode methods on acid and alkaline buffer solutions up to about  $P_H$  8.0. From about  $P_H$  8.0 to 9.0—as high as the investigations were carried—the quinhydrone electrode potentials drifted gradually.

Results obtained on buffer solutions with the quinhydrone electrode showed that gold-foil electrodes were preferable to platinum.

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# NODULAR LESIONS IN THE SPLEEN OF SWINE CAUSED BY ACTINOMYCES INFECTION<sup>1</sup>

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## ACTINOMYCOSIS IN DOMESTIC ANIMALS

Actinomycosis is a well-known, chronic, infectious disease of rather common occurrence in cattle, but is encountered less frequently in swine and other domestic animals. The infective agent, *Actinomyces bovis*, or ray fungus, may gain entrance through injuries of mucous membranes, particularly of the mouth or through abrasions of the skin. The lesions are usually localized but may become generalized.

In hogs the primary lesions are usually found in either the tonsils or the mammary glands. Occasionally the infection gains entrance through castration wounds of pigs.

Recorded cases of actinomycosis of swine involving the spleen or other internal organs are not very numerous. Zietzschmann (8)<sup>2</sup> describes a generalized case but does not state whether the spleen was affected. Knoll (5) observed a case in a pig involving the lungs, liver, and other tissues, but apparently no splenic lesions were noted. Carl (2) mentioned a case of actinomycosis in an 8-month-old pig in which the lungs, liver, stomach, and spleen were affected. Among a number of cases reported in swine by Assmann (1) there was one animal which showed lesions of the mammary glands, lungs, and spleen.

## SOURCE OF MATERIAL

For a number of years veterinary inspectors of the meat-inspection service, Bureau of Animal Industry, while making routine post-mortem inspections at slaughtering establishments, have found from time to time isolated cases of nodular lesions of the spleens of swine in which no lesions were observed in other organs or tissues. This apparent absence of lesions in the carcass, other than those observed in the spleen, and the fact that the lesions simulate somewhat those of tuberculosis, have served to stimulate rather unusual interest in these cases.

After making a histological examination of a number of such nodular spleens, received at the pathological laboratory for diagnosis, the writer found in sections from one of them a number of small, red-staining bodies showing morphological characteristics of *Actinomyces bovis*, or ray fungus.

Following these findings a special effort was made to obtain as many specimens of the nodular spleens as possible from hogs showing no other lesions, in order to determine the frequency of occurrence of the actinomycoticlike bodies in the nodules and their possible relation from an etiological standpoint.

<sup>1</sup> Received for publication July 26, 1927; issued December, 1927.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 841.

It was the intention at first to make only a histological study of the spleens, but after examining a number of specimens, several of which showed bodies simulating actinomyces, it was decided also to culture a number of the spleens in the hope that a bacteriological examination would aid somewhat in establishing more definitely the causative factor in these cases.

The observations recorded in this paper have extended over a period of several years, during which at least 50 typical specimens of the nodular spleens have been received at this laboratory. A number of the specimens were forwarded for diagnosis in routine manner from various bureau stations; others were obtained by request through inspectors in charge at the large hog-slaughtering establishments. Most of the specimens were examined histologically but only about one-half of them were cultured.

#### GROSS APPEARANCE OF THE NODULAR SPLEENS

The nodules in the different spleens vary considerably in size. Some are little larger than the head of a pin, while others may attain the size of a hazelnut. More frequently they are about the size of a pea or a little larger. The more superficial nodules were usually slightly elevated above the surface of the organ.

While on casual observation the nodular lesions in the gross specimen are somewhat suggestive of tuberculosis, a more minute examination reveals a rather distinct difference. The spleen does not show the extensive fibrous changes seen in tuberculosis. The nodules consist of a homogeneous material heavily encapsulated. The necrotic centers are of a cheeselike consistence in the younger lesions, and are usually of a greenish or greenish-yellow color. Frequently these necrotic masses can be readily detached and removed from the surrounding capsule. There is a tendency to calcification in the older lesions. The splenic pulp usually shows no visible changes outside the encapsulated nodules.

#### HISTOPATHOLOGY

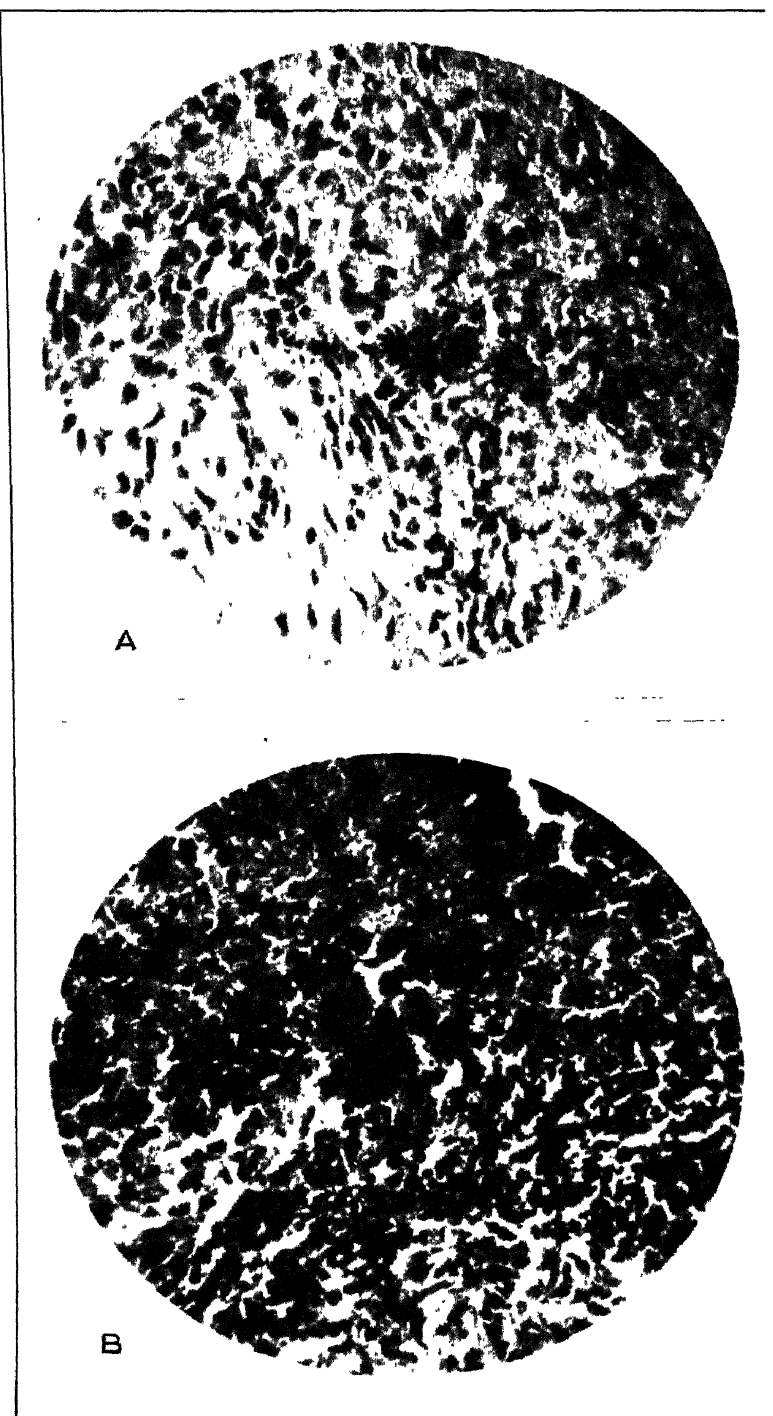
Sections from affected spleens show the lesions to consist of necrotic centers, varying in size and usually heavily encapsulated. The necrotic centers are surrounded by zones of round cells, epithelioid cells, and leucocytes. The degenerated centers of the nodules are frequently seen to be undergoing calcification. In most cases little change was noted in the splenic tissue outside of the encapsulated areas.

The actinomyces have been definitely demonstrated in seven different specimens, or 14 per cent of the spleens examined. (See pls. 1 to 5.) In other spleens degenerated bodies were also seen which were thought to be actinomyces, but they could not be definitely recognized as such on account of the poor staining resulting from degenerative changes. The undegenerated colonies, or those which had undergone only slight degenerative changes, were found to be positive to Gram's stain.

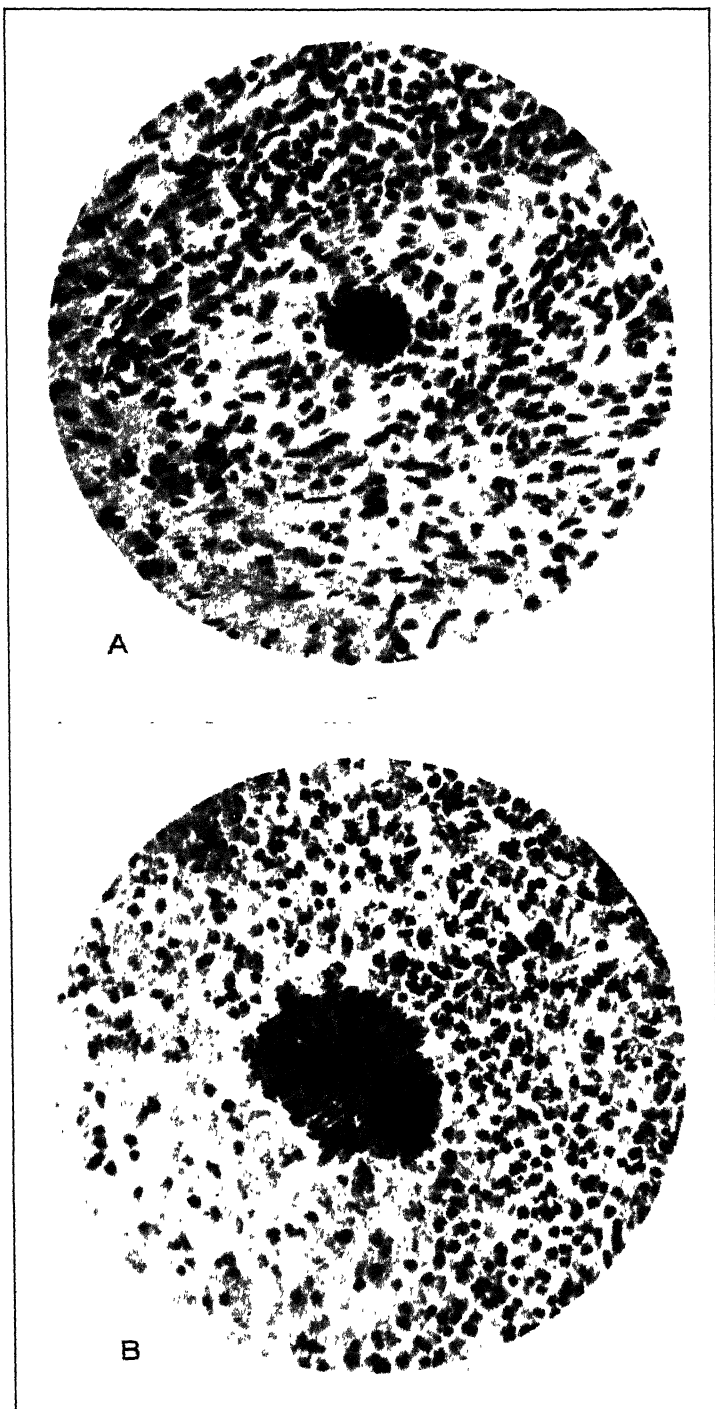
When the red-staining bodies were first observed in the swine spleens it was noted that they did not appear to be altogether typical of *Actinomyces bovis*. It is now believed that the slight morpho-



A.—Photomicrograph of section of spleen, case 5-D, showing the only colony of the organism found in this specimen.  $\times 400$   
B.—Photomicrograph of section of spleen, case 6-D. A number of small bodies similar to the one here shown were found in this case.  $\times 400$

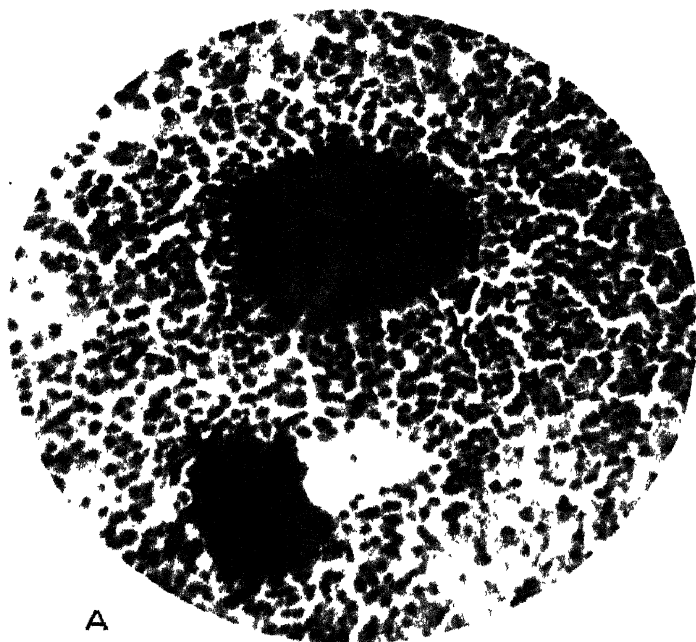


A.—Photomicrograph of section of spleen, case 9-D. The small colony here shown was the only one found in the various sections examined.  $\times 400$   
 B.—Photomicrograph of section of spleen, case 7-E, showing the only colony that could be definitely recognized in this case.  $\times 400$

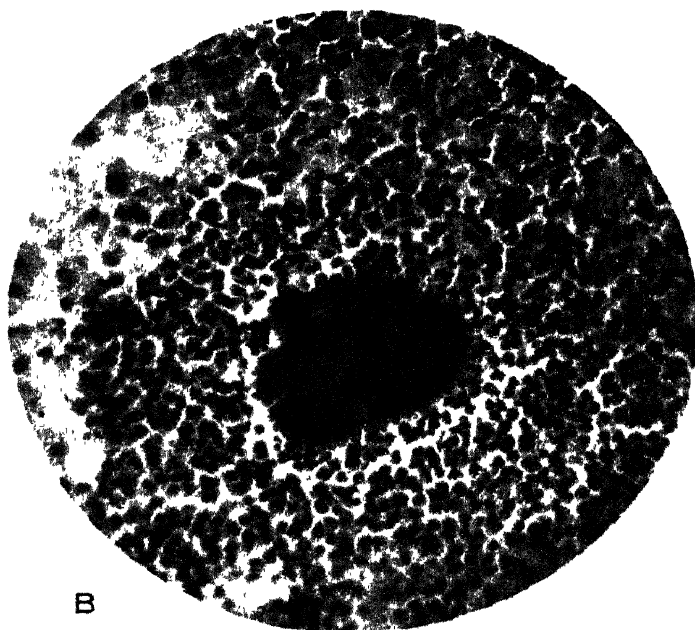


A.—Photomicrograph of section of spleen, case S-F, showing the only colony found in this case.  $\times 400$

B.—Photomicrograph of section of spleen, case 9-F. A considerable number of colonies quite characteristic of *Actinomyces bovis* were found in this specimen.  $\times 400$

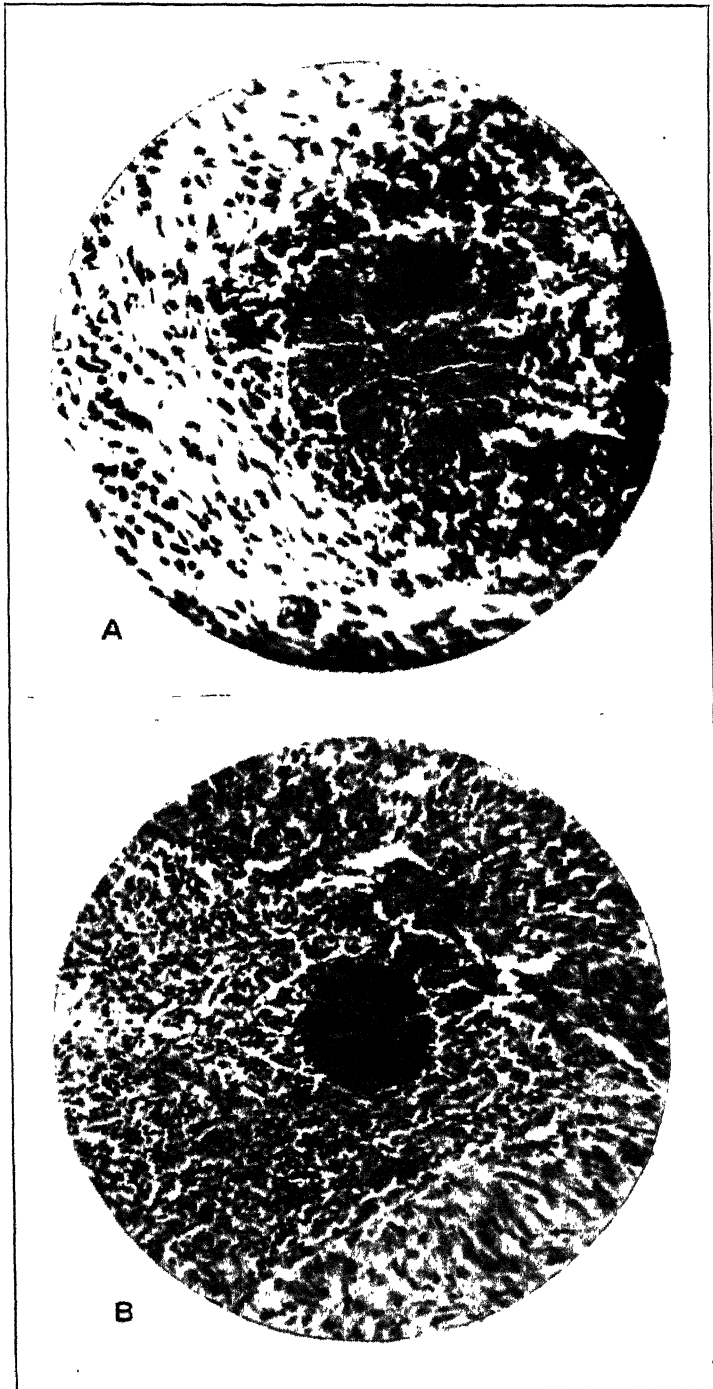


A



B

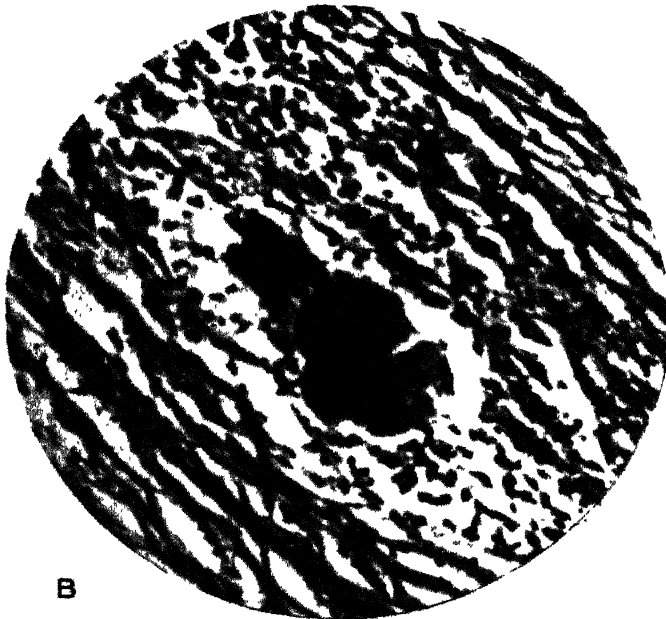
A and B.—Photomicrographs of sections of spleen, also from case 9-F, showing two more of the rather typical colonies found in the different nodules in this specimen.  
× 400



A and B.—Photomicrographs of sections of spleen, case 2-R. Practically every nodule in this spleen contained one or more colonies.  $\times 350$  and  $\times 250$ , respectively



A



B

A.—Photomicrograph of section of stomach, case 13-D, received from L. E. Day, showing colonies of the organism in lesions of stomach wall.  $\times 400$   
B.—Photomicrograph of section of stomach, case 1-P, showing a partially degenerated colony in center of the stomach wall.  $\times 350$

logical variations noted were the result of degenerative changes, as somewhat similar alterations have been observed in the organisms in cases of actinomyces infection of bovine tissues. Doubtless there had been more or less degeneration of the actinomyces even in those cases in which they were definitely recognized, and this is thought to account largely for the fact that growth of the organism was obtained in only one of the various specimens cultured.

In order to show the marked similarity of the pathological changes in the nodular spleens the following brief description is given of the lesions found in a number of the specimens, which are more or less typical of all.

CASE 3-D.—In this case there were several rather large necrotic areas surrounded by zones of leucocytic and round-cell infiltration, the whole being encapsulated. There were also a few small necrotic foci. No changes were observed in the splenic pulp outside of the encapsulated areas.

CASE 4-D.—Sections show a few small, necrotic foci which were rather heavily encapsulated and undergoing calcification. Accumulations of round cells and leucocytes are seen within the capsules, and in several places there were fusions of epithelioid cells simulating giant-cell formation.

CASE 5-D.—Nodules showed large necrotic centers surrounded by zones of cells beneath the heavy capsules. There is considerable calcification of the nodules. In one of the sections a small, rather well-defined body, quite suggestive of actinomyces, was observed, being the only one found in this case. (See pl. 1, A.)

CASE 6-D.—Nodules with necrotic centers showing borders of round cells inclosed in thick capsule. Sections from this specimen show several of the actinomycoticlike bodies, mostly within the necrotic areas. There were also a number of degenerated and partially degenerated colonies in the different sections examined. (See pl. 1, B.)

CASE 9-D.—Sections from this specimen show a number of small necrotic foci massed together and surrounded by a fibrous wall. Excessive numbers of eosinophiles were noted in the cellular infiltrations. At the border of one of the necrotic areas one of the small bodies simulating actinomyces was noted. (See pl. 2, A.)

CASE 11-D.—Practically all the nodules in this case were undergoing calcification. Within the encapsulated areas were numerous degenerated and partially calcified bodies which in size and outline were rather suggestive of actinomyces. A few of these bodies took the eosin stain but were not clearly enough defined to be identified.

CASE 1-F.—Lesions consisted of a number of small, thickly encapsulated nodules. Practically the entire encapsulated contents of the various nodules had undergone calcification. There were no changes in the spleen outside of the encapsulated areas.

CASE 3-F.—In this specimen there were a few large and a number of small nodules in different stages of degeneration and calcification, all well encapsulated.

CASE 7-F.—Each of the sections in this case contained one fair-sized nodule, encapsulated, and showed the usual necrotic and calcified centers. In one of the sections a rather characteristic actinomycoticlike body was observed. A partially degenerated colony was also noted in one other section. (See pl. 2, B.)

Therefore, after taking into consideration the difficulties experienced by others in their attempts to produce actinomycosis by culture inoculations, it is not altogether surprising to find the inoculation results here reported negative.

#### POSSIBLE MODE OF INFECTION

The fact that the lesions in practically all of these cases appear to be confined to the spleen has given rise to considerable speculation as to the manner in which the infective agent finds its way to that organ. Herzog (4) is of the opinion that the ray fungus is spread by way of the lymphatic circulation. If we consider the close proximity of the spleen and stomach, and the lymphatic relation of these two organs, it does not appear unreasonable to assume that the infection in these cases first finds lodgment in the stomach, either by simple absorption or actual infection of the stomach wall, and eventually reaches the spleen either by contiguity or through the lymphatic circulation.

During the time that these observations were being made two cases of actinomycotic infection of the stomach of swine came to the writer's attention. One of these was diagnosed by L. E. Day at the branch pathological laboratory, Chicago, Ill., and the other was received for diagnosis at the pathological laboratory, Washington. In both instances the stomach wall was very much thickened and contained fibrous nodules, one of the cases showing necrotic and calcified centers.

In the first-mentioned case the red-staining fungus growths can be readily seen in the lesions. (Pl. 6, A.) In the other the bodies are very much degenerated. (Pl. 6, B.) In both instances the lesions involve practically the entire stomach wall in places.

While the writer has no knowledge that the spleen was involved in either of these cases of stomach infection, there is of course the possibility that there might have been slight lesions in the deeper structures of the spleen which could not be readily detected in the macroscopical examination. However, regardless of such a possibility, the demonstration of these cases of infection of stomach wall strengthens the theory that the most probable way in which the infection may reach the spleen is through the stomach.

It is likewise true that in some of the animals showing nodular spleens there may have been associated stomach lesions which were slight in nature, rendering them inconspicuous on post-mortem examination. Such lesions being quite unusual might easily have been overlooked by the inspector making the post-mortem inspection.

Feldman (3) reports a case of actinomycotic infection of the omentum and peritoneum of a cow in which all other organs and tissues were free from the disease. He was at a loss to account for the source of entry of the causative organism. This case is mentioned simply to illustrate how the actinomyces may reach an internal organ or tissue without leaving any visible trace to indicate its path.

#### APPARENT RESISTANCE OF AFFECTED ANIMALS

As a large percentage of hogs are slaughtered before reaching 1 year of age, most of the specimens of nodular spleens examined were obtained from animals ranging from 6 to 12 months of age. The

chronic character of the lesions indicates that the pigs probably become infected during the first few months of life. The resistance of the animal to this splenic type of infection is evidenced by the heavy fibrous wall thrown around the infected centers, and the sterile condition of the encapsulated necrotic material in a large percentage of cases. This would account for the presence of the actinomyces in only a small percentage of the cases examined, and as many of the colonies seen in sections were found to be undergoing degenerative changes it probably also accounts for the fact that cultures of the organism could be obtained from only one of the various specimens cultured. In other words, it would seem that the finding of the living or undegenerated organisms in these cases is rather difficult and depends largely on the age of the lesions at the time the hog is slaughtered.

### CONCLUSIONS

The similarity of the lesions, both macroscopically and microscopically, in the cases investigated is indicative of a common etiological factor.

The fact that no other possible causative factor was found in the lesions, and that *Actinomyces bovis* was recognized in at least seven different specimens, seems sufficient to identify this organism as the cause of the splenic lesions.

The character of the lesions and the large percentage of cases in which the causative organism was no longer present indicate that the pigs probably become infected early in life, and that they are quite resistant to such splenic infections.

The evidence at hand points to the stomach as the most probable portal of entry through which the actinomyces eventually reach the spleen.

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# A MODIFICATION OF BRUCE'S METHOD OF PREPARING TIMBER-YIELD TABLES<sup>1 2</sup>

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In Bruce's method of preparing timber-yield tables<sup>2</sup> anamorphosis is used for constructing the curves for site classification, total basal area, average basal area, number of trees, and total cubic volume. Since, by construction, each set of curves is represented by a series of straight lines radiating from a common point, it is evident that the reading of any two lines must have the same ratio at each age. Any curve of such a series can therefore be expressed as a percentage of any other curve of that series, such as the average curve. This attribute of anamorphically constructed series of curves<sup>3</sup> permits their expression in the form of alignment charts, the use of which eliminates the laborious anamorphic plotting of the data and the balancing of a curve for each site-index<sup>4</sup> class, results in increased accuracy, and makes interpolation and checking easier.

The first step in constructing these alignment charts is the preparation, for site classification, of an average curve<sup>5</sup> over age for—

- (1) Height of average dominant tree;
- and, for the entire stand, similar curves for—
  - (2) Total basal area, hereafter called stand basal area.
  - (3) Average basal area, hereafter called tree basal area.
  - (4) Number of trees.
  - (5) Volume in cubic feet.

These curves are similar to the graduating curves used by Bruce, but may be drawn in the conventional way rather than as graduating curves. The average points for the tree basal area curve should be computed from the averages of stand basal area and the numbers of trees used for constructing the average curves for these two factors, instead of using averages of tree basal areas of individual plots; otherwise the final curves for tree basal area will not balance.

The curves for tree basal area, numbers of trees, and stand basal area are checked against each other; at any age the product of tree basal area and number of trees should equal the stand basal area.

<sup>1</sup> Received for publication June 25, 1927; issued December, 1927.

<sup>2</sup> BRUCE, D. A METHOD OF PREPARING TIMBER-YIELD TABLES. Jour. Agr. Research 32:543-557, illus. 1926. Errata: Figures 7 and 8 should be interchanged and the applied stand graph should be referred to Figure 7 instead of Figure 8.

<sup>3</sup> This applies, of course, only to systems of curves having a common origin.

<sup>4</sup> "Site index" is the height which the average dominant tree will have, or had, at some standard age, usually 50 (or 100) years.

The site-index system should not be considered as a banding system comparable with but more finely subdivided than the ordinary "I, II, III" or "Good, medium, poor" system. Rather, the farmers' classification of farm land as 45-bushel wheat land (in 1 year implied), or 24-bushel corn land (1 year) is entirely comparable with 93-foot (50-year) longleaf pine land. The only difference is that farm-land quality is rated by actual yield, but forest-land quality, because of the variation in yield with density of stocking, must be rated by measurement of some other factor (height) indicative of the productive capacity, but for the most part not appreciably affected by variations in density.

The grouping of plots into 10-foot site-index classes is done only for convenience in preparing the curves, and in the final tables values are given for only the even 10 feet of site index to avoid bulkiness. Values for every foot of site index, or even smaller intervals, could be determined, but intervals less than 1 foot are not practicable.

<sup>5</sup> These curves should be carefully balanced so that the sums of the positive and negative deviations are equal and distributed as evenly as possible throughout the length of the curve.

In addition to the curves listed above a so-called average *forest form factor-age* curve is drawn for checking purposes. This "forest form factor" is obtained by dividing the cubic volume by the product of stand basal area and the dominant heights<sup>6</sup> used for the site classification (*height-age*) curve.

Age-class averages of cubic volume, height, and stand basal area should be used to derive the average forest form factor for each class instead of computing the form factor for each plot and then averaging the form factors by age classes. The curve thus derived is used to check the stand basal area, height, and cubic volume curves against each other. At any age the volume should equal the product of stand basal area, height of the average dominant tree, and forest form factor.

A site classification alignment chart is now prepared from the *height-age* curve and a percentage alignment chart. Percentage charts may have either arithmetic or logarithmic scales. The logarithmic type gives better intersections and readings of the same relative accuracy throughout and is also less cumbersome to use. For these reasons logarithmic charts should be employed.

To construct the percentage chart lay out three parallel axes, *A*, *B*, and *C* (fig. 1), *B* midway of the others. On *A* and *C* lay out equal logarithmic scales. On *B* lay out a logarithmic scale with cycles half as large as those used on *A* and *C*, so placed that the 100 on this scale lies exactly on the line through 100 on *A* and *C*. (Only the *B* axis need be actually graduated, as will be shown later.) A line passing through any two numbers on *A* and *B* will intersect *C* at the point representing the ratio or percentage  $\frac{B}{A} \times 100$ .

To convert this chart into the site classification chart all that is necessary is to let the graduations on the *A* and *B* axes represent the height of the average dominant tree in feet, and to superpose on *A* and *C* the scales for age and site index, as follows:

On the *A* axis the ages are marked opposite the graduations corresponding to heights read from the average *height-age* curve, as listed below, resulting in scale *A*.

Age in years	Height of average dominant tree in feet	Age in years	Height of average dominant tree in feet
10	14	60	78
15	26	70	83
20	37	80	87
30	53	90	90
40	64	100	92
50	71		

Each site index expressed as a percentage of the average site index<sup>7</sup> is then marked opposite that percentage on the *C* axis, resulting in scale *C'*. This may also be done graphically by drawing, from the classification age on *A*, a series of lines to *C* passing through the 10-foot intervals on *B*. Their intersections on *C* fix the positions of the 10-foot site-index graduations. The intermediate site-index graduations may be located in the same way. The unnecessary

<sup>6</sup> The dominant height is substituted for average height to permit checking the site-index curves with the volume and stand basal area curves. Curves for average height are not constructed by anamorphosis and are therefore not susceptible to such simple checking.

<sup>7</sup> This is the height of the average *height-age* curve at the classification age (50, 100, etc., years).

logarithmic (height and percentage) graduations on *A* and *C* can now be erased, leaving only the age graduations on *A'*, the heights on *B'*, and the site indices on *C'*. A line through the age of a plot

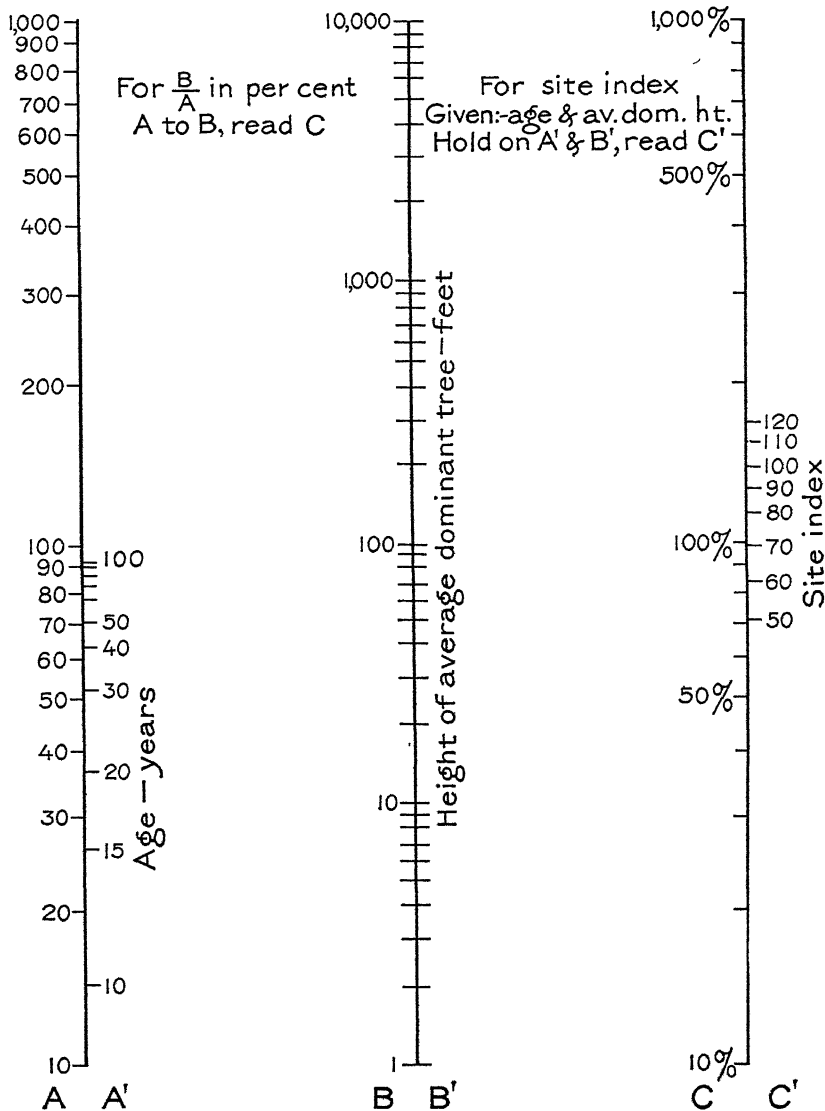


FIG. 1.—Logarithmic chart for computing percentages, converted into chart for classifying site

and the height of its average dominant tree will then intersect *C'* at the site index of that plot.

The next step in the use of this chart is the assignment of a site-index value to each plot, after which the plots are grouped by site-index classes (10-foot classes preferably).

Working first with stand basal area, an estimated stand basal area corresponding to the age of the plot is read from the average *stand basal area-age* curve. (Fig. 2, A.) Both observed and estimated

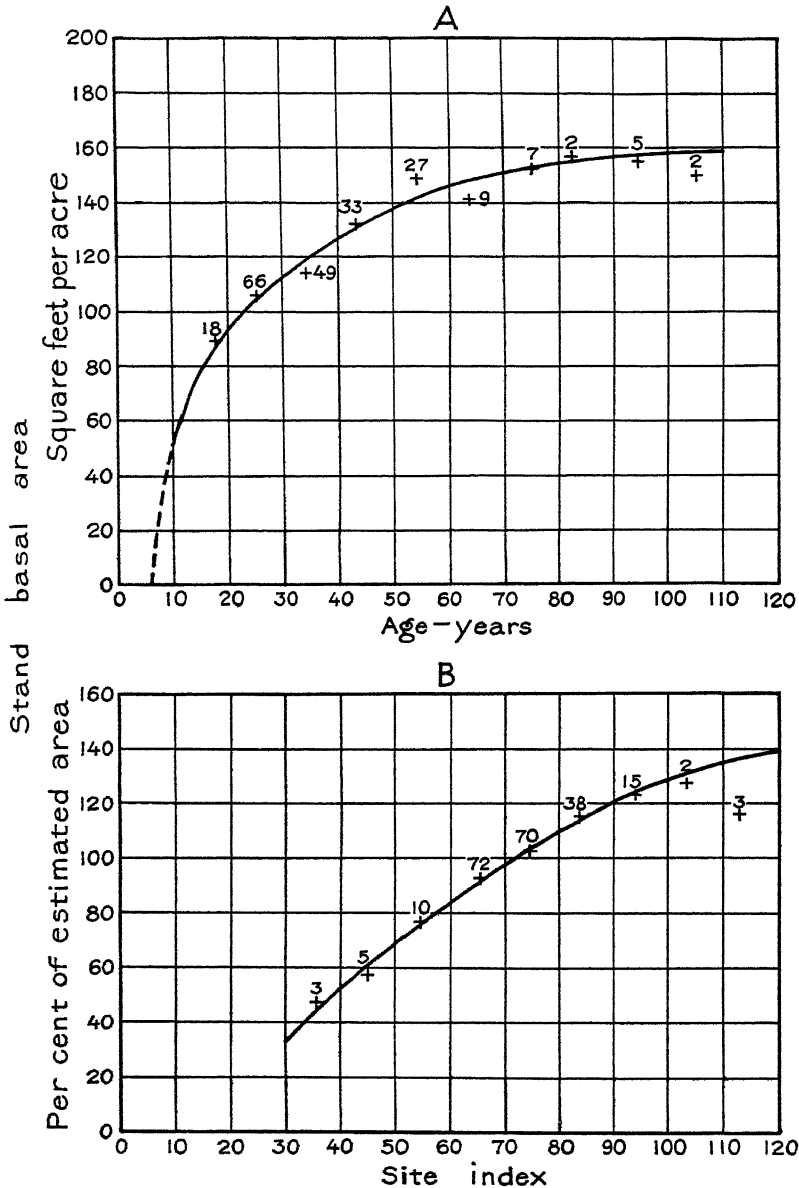


FIG. 2.—Curves used for preparing alignment chart stand basal area: A, average curve over age, used to graduate age axis in Figure 3; B, average curve over site index, used to graduate site-index axis in Figure 3

areas are then totalled by site-index classes and the sums of the observed values expressed as percentages of the sums of the estimated. Table 1 illustrates this. These percentages are then plotted over

the average site index of the class and curved, as in Figure 2, B. Similar curves are prepared for tree basal area, number of trees, and cubic volume, and are checked against each other. For each site index the percentage of stand basal area should equal the product of the percentage of number of trees and percentage of tree basal area.<sup>8</sup> The cubic volume curve could be checked through forest form factor, but ordinarily such a check is not needed, since the *volume per cent-site index* curve is usually a well-defined and straight or nearly straight line. Readings from the curve for stand basal area are then superposed on a percentage alignment chart, as was done for the site classification chart; the ages as obtained from the average *stand basal area-age* curve appearing on the *A* axis, and the site indices, from the *stand basal area per cent-site index* curve, appearing on the *C* axis. Similar charts are derived for tree basal area and diameter, number of trees, and total volume of peeled stem wood in cubic feet.

TABLE 1.—*Computations of stand basal areas, by site index classes, as percentages of stand basal area of average site index*

Plot No.	Number of plots	Site index	Age	Stand basal area		
				Observed	Estimated from average curve over age	Observed in per cent of estimated
			Years	Sq. ft.	Sq. ft.	Per cent
8.....		31	16	36	83	-----
46.....		36	24	56	102	-----
31.....		39	21	42	97	-----
Total.....	3	106	-----	134	282	-----
Average.....		35.3	-----	-----	-----	47.5
112.....		40	32	77	116	-----
80.....		41	27	46	108	-----
168.....		49	44	110	133	-----
119.....		47	34	48	119	-----
25.....		48	20	48	94	-----
Total.....	5	225	-----	329	570	-----
Average.....		45.0	-----	-----	-----	57.7
55.....		50	25	51	104	-----
153.....		57	40	86	128	-----
Total.....	10	545	-----	935	1,223	-----
Average.....		54.5	-----	-----	-----	76.5

#### SUMMARY

	3	35.3	-----	134	282	47.5
	5	45.0	-----	329	570	57.7
	10	54.5	-----	685	1,223	76.5
	72	65.2	-----	8,183	8,835	92.6
	70	74.1	-----	8,894	8,715	102.1
	38	83.4	-----	5,854	4,620	115.9
	15	94.0	-----	1,637	1,573	123.1
	2	103.0	-----	232	182	127.5
	3	113.0	-----	311	268	116.0
Total.....	218	-----	-----	26,309	26,268	-----

\* Totals are for 10 plots in this group, of which only 2 are shown.

<sup>8</sup> If the average curves over age have not been perfectly balanced these curves will not pass through 100 per cent at the average site index. Thus a simple check is combined with an automatic correction for any inaccuracies in balancing the curves drawn in the first steps.

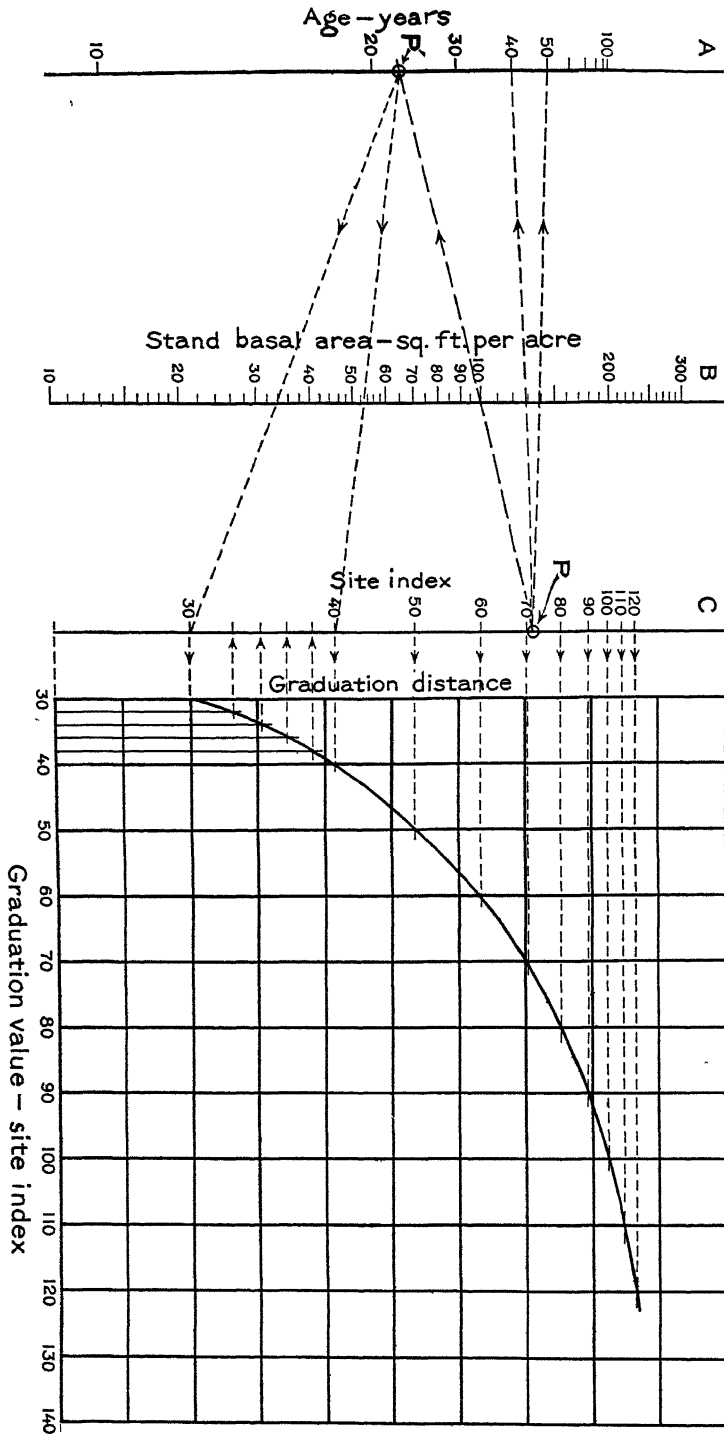


FIG. 3.—Alignment chart for stand basal area, indicating the location of the major graduations by intersection, and the preparation and use of a graduating curve for locating minor graduations for site index.

To avoid the necessity for first constructing percentage charts, a somewhat different procedure may be followed. The three parallel axes are first drawn, not necessarily at uniform intervals. On the central axis a logarithmic scale of stand basal area is entered, as in Figure 3. Any point *P* is then temporarily marked on *C* as the position of the average site-index graduation. Lines drawn from *P* and intersecting *B* at values read from the average *stand basal area-age* curve will intersect *A* at the location of the corresponding age graduations.

For locating the site index graduation on *C* the graduations of *B* are temporarily considered as percentages, and a line from *P*, through 100 per cent, locates a point *P'* on *A*. Lines from *P'*, intersecting *B* at percentages, read from the *stand basal area per cent site-index* curve, will intersect *C* at the position of the corresponding site-index graduations.

Charts for each of the other factors are similarly constructed. The chart for tree basal area can be read directly in terms of average breast-high diameters by marking the diameters along the *B* scale where the corresponding basal areas appear, as in Figure 4. Percentages for tree basal area should be computed from totals of stand basal area and number of trees by site-index classes.

This chart for diameters is also used for obtaining partial-stand values in terms of percentages of the corresponding total-stand values. The curves described by Bruce<sup>9</sup> are used for this.

A point (*P*, fig. 4) is permanently marked to the right of the *A'* axis. A series of axes parallel to *X* are drawn (*B*, *C*, *D*, etc.), their number depending on the number of ratios to be represented. Percentages are then marked on these axes at their intersection with lines, from *P*, through the corresponding average breast-high diameters of the total stand on *X'*. The same procedure is followed for the average diameters of the partial stands, except that the curves used by Bruce (difference between partial and total stand diameters over total stand diameter) are replaced by curves of partial-stand diameters over the entire-stand diameter.

Only a few graduations on the axis of any of the alignment charts need be located by intersection. The distances of these from any fixed point on the axis are then curved over the value of the graduation (age, site, index, diameter, etc.) and the distances of the intermediate graduations transferred from this curve. Figure 3 illustrates the location of the intermediate site-index graduations from the curve passing through the distances of the 10-foot graduations.

When the aggregate checks of the various factors are made it will be necessary in the case of tree basal area to weight the values for each plot by the observed number of trees; this is equivalent to comparing the observed stand basal area with that obtained by multiplying estimated tree basal area by the observed number of trees.

By Bruce's method the fit of the radiants indicates whether or not anamorphosis can be legitimately employed in preparing the curves. A consistent failure of the points to fall into straight lines indicates that the method is not suitable.

With the modifications here presented the applicability of anamorphosis can be tested as follows: The observed values and the estimated

<sup>9</sup> BRUCE, D. Op. cit. p. 549, fig. 5; p. 551, fig. 6.

values corresponding to the age and site index of each plot are grouped, summed, and averaged by 10-year age—10-foot site-index classes. The deviations of the observed averages from the estimated aver-

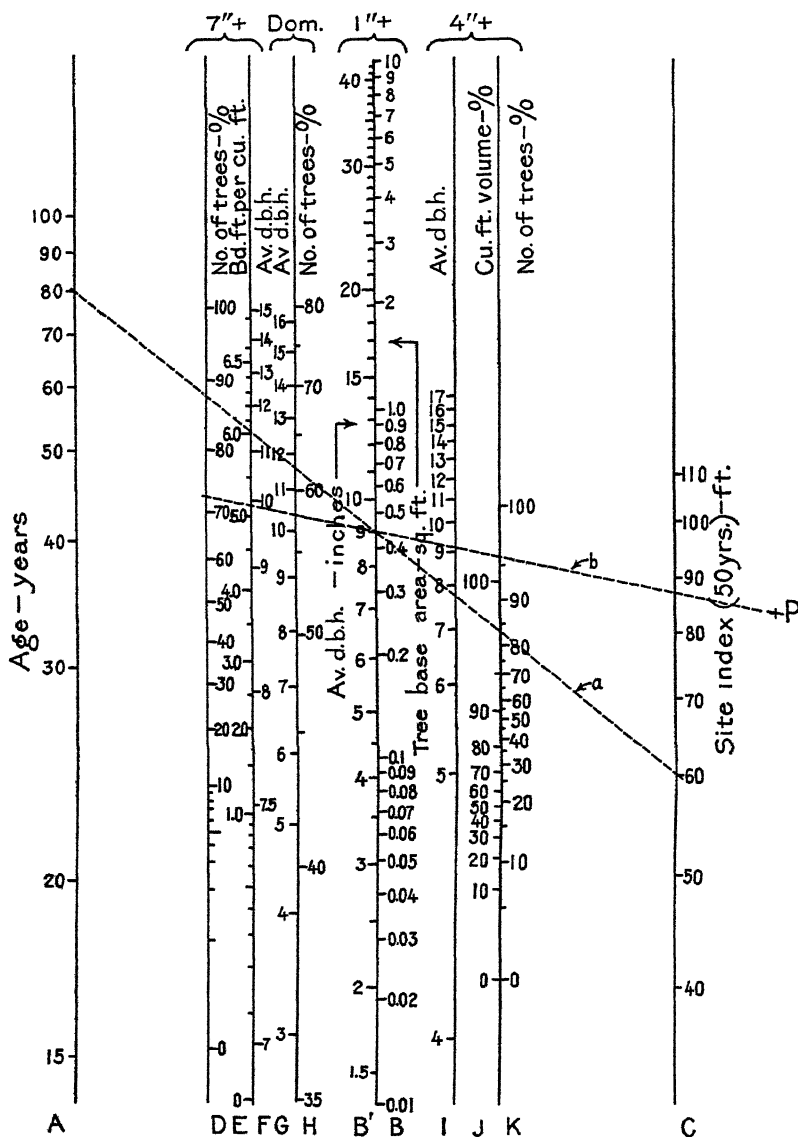


FIG. 4.—Alignment chart for tree basal area with scale reading in terms of average d. b. h. ( $B'$ ). The additional axes,  $D$  to  $K$ , are graduated to show the partial stand values in per cent of the total stand values when a straight line is passed through  $P$  and the average d. b. h. of the total stand

ages are plotted over age by site-index classes. Failure of the method to fit will be indicated by a series of progressively changing curves. This is not the case with stand basal area in the illustrative example as shown in Figure 5. Such a test, so essential in the development

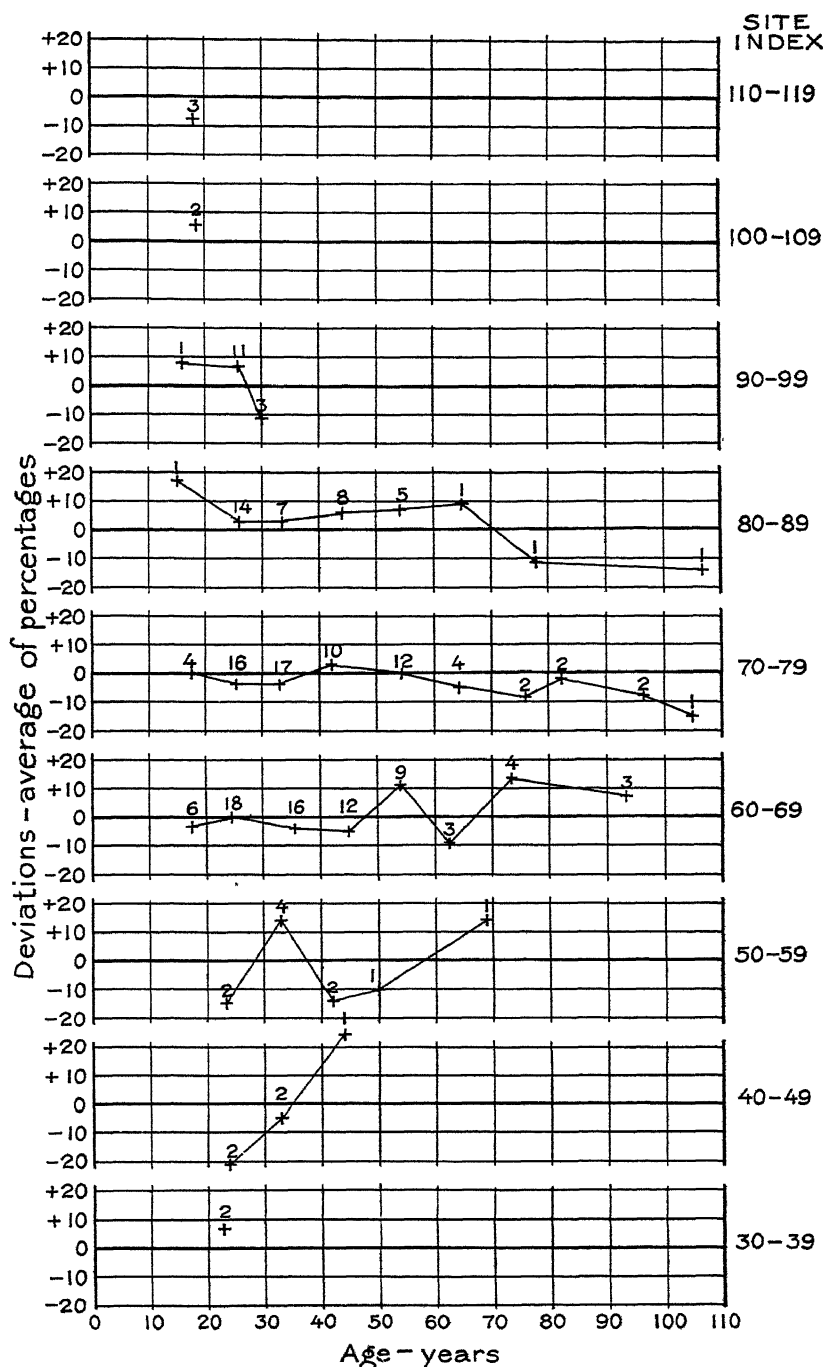


FIG. 5.—Percentage deviations of stand basal area plotted over age, by 10 foot site-index classes, showing that no consistent departure from the chart values exists, thus proving that the method used introduces no distortion

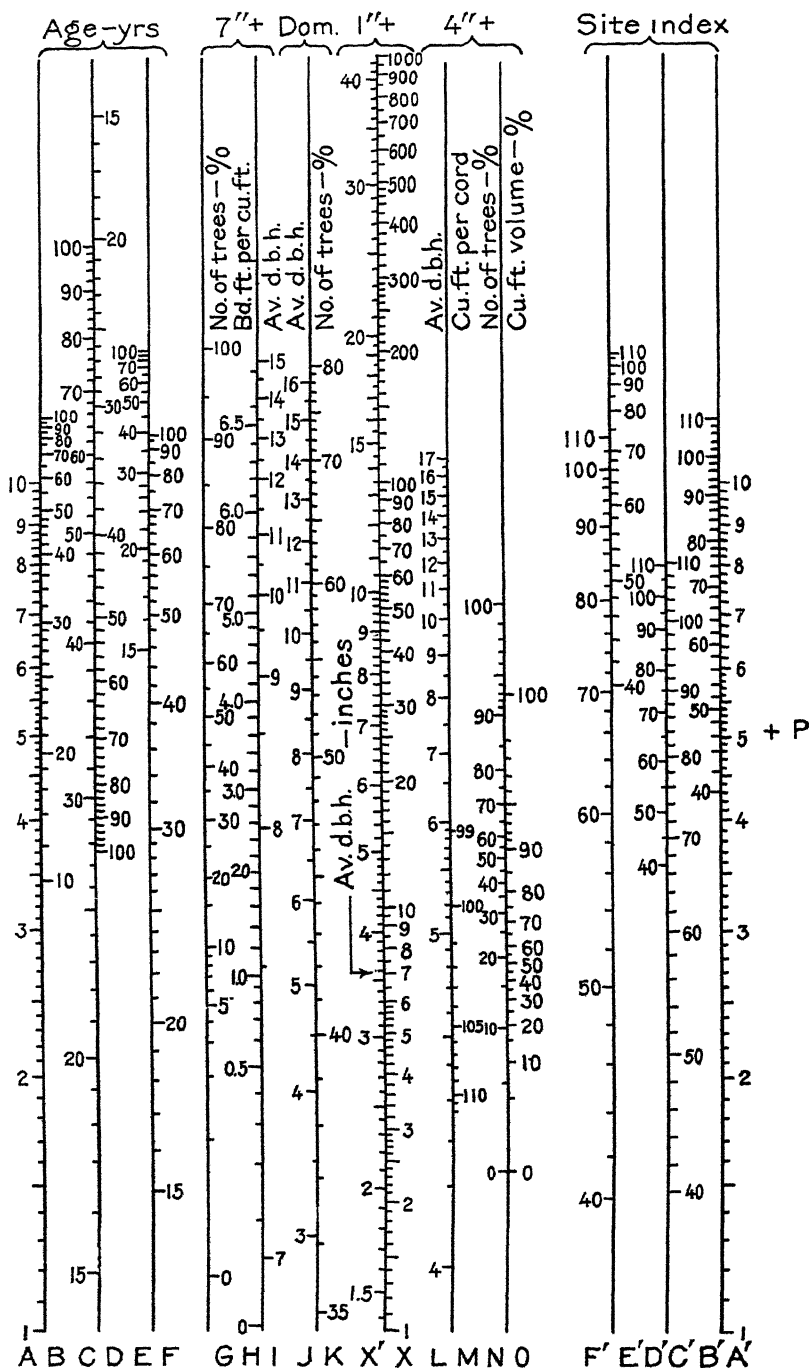


FIG. 6.—Composite chart for total-stand and partial-stand values. All total-stand values are read on the central scale. The two outer scales, *A* and *A'*, are used with *X* for multiplying total-stand values by partial-stand percentages to get partial-stand values. (See appendix for instructions for using this chart)

of a new technic, is of little value now. Experience with nine species of diversified characteristics has justified the assumptions basic to the method.

Separate charts need not be made for each factor; they may be combined into a single chart by using a common central axis for all factors and separate outside axes for each factor, as shown in Figure 6. In this case the readings on the central logarithmic scale are multiplied or divided by 10 or 100, as necessary for the factor considered.

An additional pair of scales used in conjunction with the central

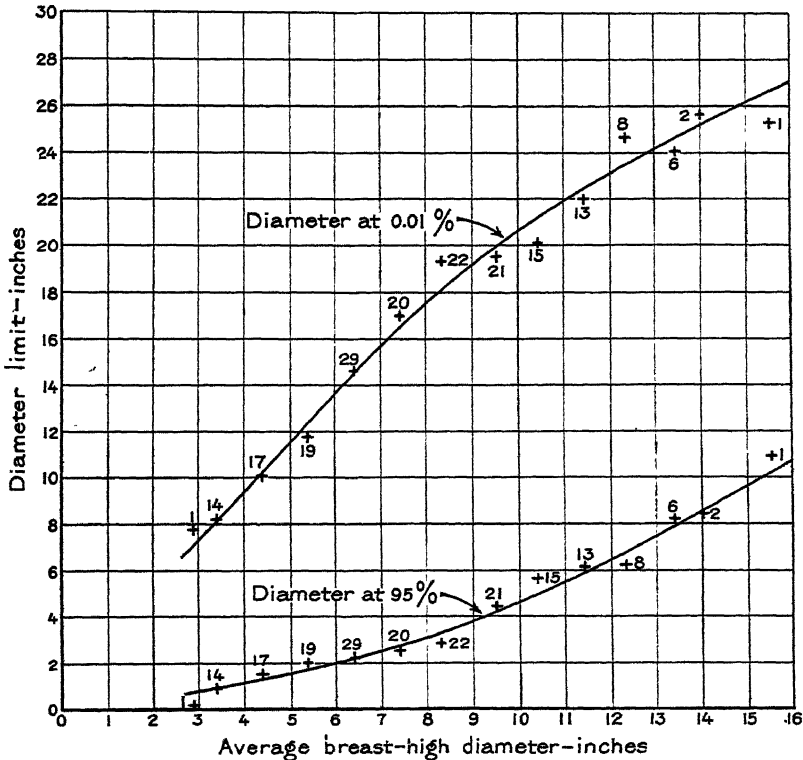


FIG. 7.—Intercept curves for stand-table graph. The diameters at which the basic frequency curves intercept the 95 and 0.01 ordinates are plotted over the average diameters (by basal area) of the respective frequency curves. The curves fitted to these points are used to construct the *A* and *B* scales of Figure 8

one can be profitably added to this chart for multiplying total stand values by percentages to get partial stand values (*A* and *A'*, fig. 6). These will be ordinary logarithmic scales with cycles matched with those of the central scale.

Stand tables may also be included by using a series of percentage scales, but a more usable and simpler way of presenting them is by means of the stand-table graph employing "frequency" paper where such paper fits the data. In a number of cases the arithmetic paper specifically described by Bruce has failed to represent the data by straight lines. With one species the logarithmic type was resorted to in order to straighten the frequency curves. For another species neither type of paper gave satisfactory results at first. Straight

lines were finally obtained by plotting the percentages on logarithmic paper over the diameter *plus a constant*. This constant was the same for stands of any average diameter. For still another species the constant added varied with the average diameter of the stand, the

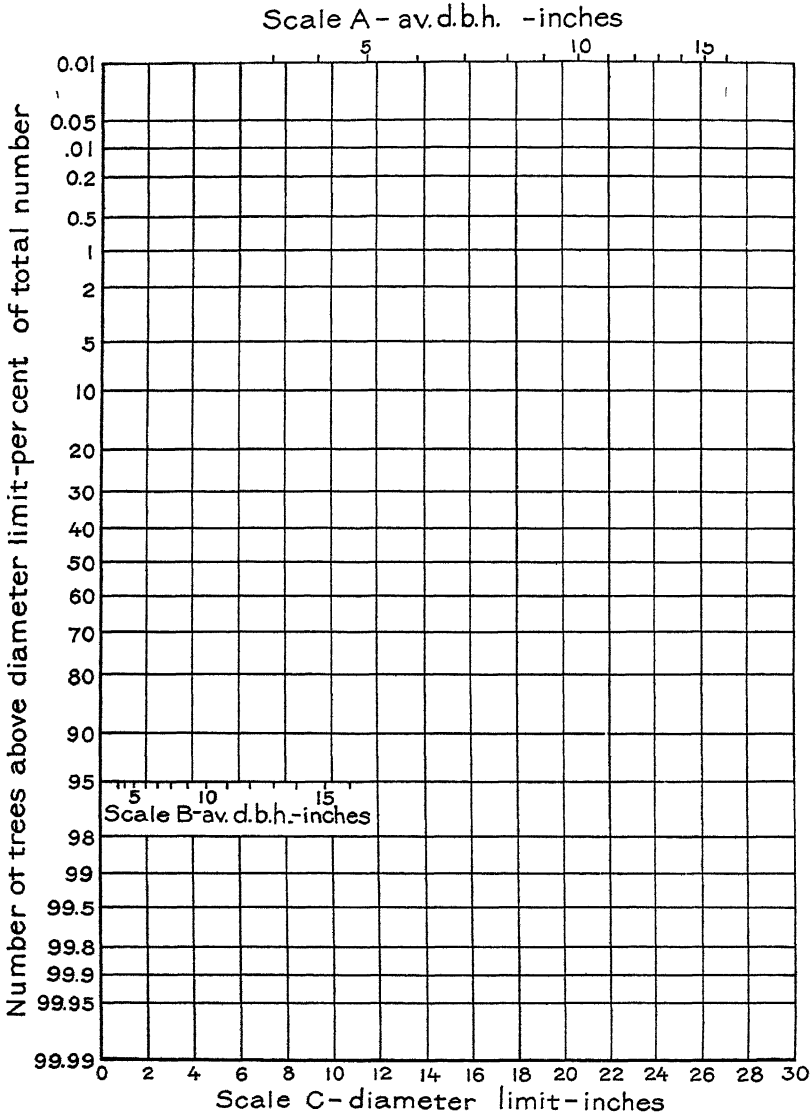


FIG. 8.—Stand-table chart. The frequency distribution in a stand will be shown by a straight line passing through the A and B scales at the average diameter (by basal area) of the stand

best results being obtained when the constant added for each stand was the same as its average diameter.

The pair of charts used by Bruce for presenting the stand tables graphically can be simplified and combined in one chart, easier to use, by substituting two additional scales to take the place of his

two curves of standard deviation and average diameter. To do this, two somewhat similar curves are prepared from the readings of the basic frequency curves<sup>10</sup> at percentages as far apart on the chart as convenient—in the example given here the readings of 95 and 0.01 per cent are used. The diameters at which each frequency curve intercepts the two percentage lines selected are plotted over the average diameter for this frequency curve. A curve is fitted to each of the two series of points, as shown in Figure 7. Reading back from these two curves, two scales of average diameter are constructed along the percentage lines. These are scales *A* and *B* in Figure 8. To locate the line representing the frequency distribution in a stand of given average diameter all that is necessary is to draw a line passing through this average diameter on both of the *A* and *B* scales.

It will be noted that the frequency graph presented here has percentages along the vertical scale and diameters along the horizontal scale, in keeping with the standard practice of using abscissas for the independent variable and ordinates for the dependent variable. The frequency paper as printed does not conform to this standard.

#### SUMMARY

The modification here reported of Bruce's method of preparing timber-yield tables consists, chiefly, in expressing the conventional system of curves by means of alignment charts. The curves developed by Bruce's method may be converted into alignment-chart form, but several advantages accrue from deriving the alignment charts directly from the basic data. Chief among these advantages are the reduction in the number of curves to be fitted, with a consequent increase in definition of each curve, and the saving in labor made possible by the ease of interpolating the alignment charts for any age and site index. In addition to the saving of labor in preparing the yield tables the alignment-chart form permits the presentation of final results in greatly condensed form; a single sheet of pocket notebook size is sufficient to carry the results with enough accuracy for field use.

To insure greater accuracy an additional cross check between height, stand basal area, cubic volume, and "forest form factor" has been incorporated. Several minor errors in Bruce's method have been corrected. A simpler method of computing and graphically presenting stand tables has been described.

The method, as modified, has no disadvantages not inherent in Bruce's method, except that partial-stand values can not be read directly but must be obtained through converting factors. It has all the advantages of Bruce's method plus the advantages resulting from the use of alignment charts.

<sup>10</sup> The basic curves should be prepared from the data grouped by average-diameter classes, instead of by age-site index classes as used by Bruce. No variation in form of the curves has been detected between stands of the same average diameter but of different age or site quality. The age-site index grouping was desirable, in technic development, for analysis. Since the soundness of the technic has been established, however, it is more desirable to use the grouping by average diameter, with its resultant stronger, fewer curves.

## APPENDIX

## INSTRUCTIONS FOR USING ALIGNMENT CHART YIELD TABLE

For—	Hold age on—	Hold SI on—	Read—	Multi- ply by—
A. For site classification hold age on <i>B</i> , hold height of average dominant on <i>X</i> , and read site index on <i>B'</i> .				
B. Height of average dominant.....	B	B'	X	-----
C. Entire stand:				
1. Average d. b. h., inches.....	C	C'	X'	-----
2. Tree basal area, square feet.....	C	C'	X	0. 01
3. Number of trees per acre.....	D	D'	X	10
4. Basal area, square feet per acre.....	E	E'	X	-----
5. Volume, total, less bark, cubic feet per acre.....	F	F'	X	100
D. Partial stand: For any of the five factors listed under <i>C</i> determine its entire-stand value first. Determine average d. b. h. ( <i>C-1</i> ) also. Pass a line through this average d. b. h. (on <i>X'</i> ) and the point <i>P'</i> .				
1. For the stand 4 inches plus—				
Read average d. b. h. on.....	L			-----
Read per cent number of trees on.....	N			-----
Read per cent volume (cubic feet) on.....	O			-----
Read cubic feet per cord on.....	M			-----
2. For the stand 7 inches plus—				
Read average d. b. h. on.....	I			-----
Read per cent number of trees on.....	G			-----
Read board feet per cubic foot on.....	H			-----
3. For the dominant stand—				
Read average d. b. h. on.....	J			-----
Read per cent number of trees on.....	K			-----

Multiply the entire-stand values by the percentages and ratios read, holding the entire-stand value on *A*, the percentage or ration on *A'*, reading the partial-stand value on *X* pointing off as with a slide rule.

NOTES.—The cubic feet per cord values (*M*) represent the ratio of cubic feet (entire stem, less bark) to cords (to 3-inch top d. i. b.)

The board feet per cubic foot values (*H*) represent the ratio

$$\frac{\text{Board feet stand 7-inch plus}}{\text{Total cubic feet entire stand.}}$$

# THE SUPPLEMENTARY RELATION BETWEEN THE PROTEINS OF CORN AND OF TANKAGE DETERMINED BY METABOLISM EXPERIMENTS ON SWINE<sup>1</sup>

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## INTRODUCTION

High-grade tankage, or meat meal, is one of the most effective supplements to corn in the growing and fattening of swine. The tankage supplements the corn (1) by contributing calcium to a feed that is exceptionally low in this essential mineral, and (2) by supplying a large quantity of protein to a feed comparatively deficient in this material.

This paper is concerned with the protein relation between corn and tankage, and particularly with the question whether the effect of tankage proteins in this combination is due only to their intrinsic value in nutrition, or whether it is also due to a supplementary relation with the proteins of corn, by which each source of protein increases the utilization of the other in anabolism. The problem evidently requires the biological evaluation of the proteins of corn and tankage alone and in combination by the use of quantitative methods.

For reasons that have been discussed fully elsewhere (8),<sup>2</sup> it appears that the most direct method of studying the biological efficiency of the proteins from different food materials is the nitrogen metabolism experiment, in which the wastage of dietary nitrogen in digestion and metabolism can be determined from the nitrogen excretion in feces and urine as compared with the excretions on a nonnitrogenous diet. It is true that the values thus obtained relate to the total nitrogen or the crude protein of the food material rather than to the true protein; however, it appears that the nonprotein nitrogenous constituents of foods should not be disregarded in determining their protein values (12), even though it were possible to do so in such experimental investigations.

## PREVIOUS INVESTIGATIONS

A number of investigations of the nitrogen metabolism of growing pigs receiving protein from individual feeds or from definite combinations of feeds have been reported by McCollum (6) and by Hart and Steenbock (3) from the Wisconsin Agricultural Experiment Station. In these investigations the biological efficiency of the protein fed is measured by the nitrogen balance, expressed either as a percentage of the nitrogen intake, or as a percentage of the absorbed nitrogen, determined in the usual way. While these measures may possess a good deal of practical significance, their scientific value is impaired by the fact that the nitrogen balance does not represent the total retention of nitrogen from the nitrogen consumed or absorbed,

<sup>1</sup> Received for publication Aug. 4, 1927; issued December, 1927.

<sup>2</sup> Reference is made by number (italics) to "Literature cited," p. 863.

since it neglects the nitrogen used for maintenance. In replacing nitrogen losses from the body occurring as a result of the endogenous katabolism, dietary nitrogen is serving in a substitutive way, as contrasted with its additive utilization in growth, represented by the nitrogen balance. A comparison of the intake of nitrogen with the nitrogen added to the body neglects the nitrogen that is replacing body nitrogen, and hence is crediting the intake with only part of its actual usefulness in covering the protein requirements of the animal.

These measures of biological efficiency are, therefore, too low; they will vary not only with the quality of protein fed, but also with the amount consumed. The greater the consumption of a given protein the greater the proportion of it that will be used for growth and the more nearly will the nitrogen balance represent its total value to the body, and consequently the higher will the percentage "retention" become.

This objection to such measures of protein efficiency is overcome by measuring what has been called by Thomas (15) the "biological value" of the dietary protein (nitrogen). The biological value is the percentage of the absorbed nitrogen that is retained for both maintenance and growth, and hence is not eliminated in the urine. Its calculation involves indirect estimations of the food nitrogen in both feces and urine, as distinguished from excreted body nitrogen. Methods by which these estimations may be made and the assumptions necessarily involved have been discussed by one of the present authors (9). These methods, originally used in metabolism experiments on rats, have been successfully applied to pigs in the experiments to be described in this paper.

The metabolism experiments of McCollum and of Hart and Steenbock may be recomputed so as to yield estimates of the biological values of the proteins fed by assuming average values of the endogenous losses of nitrogen by pigs (8). In McCollum's experiments (6) the endogenous losses of nitrogen in the urine may be estimated from the observed excretions of creatinine nitrogen on the basis of earlier observations of this investigator (7) to the effect that the total endogenous nitrogen in the pig averages 5.5 times the creatinine nitrogen. These calculations give average biological values of 48 for corn (5 pigs) compared with 44 for wheat (4 pigs), 42 for oats (4 pigs), 67 for casein (1 pig), and 80 for milk (1 pig) on rations containing from 7 to 17 per cent of protein.

Experiments by Hart and Steenbock (3) on swine, recalculated in a less satisfactory way (8), have indicated a biological value of 60 for corn proteins. In a number of experiments on rats one of the writers has obtained (10) an average biological value of 60 for corn proteins at a 10 per cent level of intake.

The only experiments of which the writers are aware relating to the biological value of tankage were published from this laboratory over three years ago (10). In six experiments upon three rats an average biological value of 31 was obtained on a ration containing 10 per cent of tankage protein. That a low biological value may be characteristic of the proteins of several animal by-products is also indicated by other experiments of Hart and Steenbock on swine (2). Recalculating the results of these experiments on the assumption of average endogenous losses of nitrogen, an average biological value of

49 was obtained for meat crisps and 58 for fish meal from three pigs in each case.

A combination of corn proteins and tankage proteins in equal parts has been shown to have an average biological value of 62 for rats (11), somewhat higher than the value for corn alone at the same level of intake, i. e., 10 per cent. The experiments of Hart and Steenbock (2) on pigs indicate a biological value of 68 for a mixture of corn and tankage proteins in the proportion of approximately 3 to 2.

From the results on rats it appears that the proteins of tankage are of exceptionally low biological value, but that when combined with corn proteins such a marked supplementary relation exists that the mixture possesses a slightly higher biological value than the proteins of corn. The data available on swine, such as they are, tend to support this conclusion, but they are not sufficiently exact to demonstrate it. Hence an experiment on pigs, carried out in essentially the same manner as those previously reported on rats, has been undertaken with the results described below.

### METHODS

Eight young Poland-China barrows were used in this investigation. They were confined in metabolism crates very similar in construction to that described by Forbes (1), the only essential difference consisting in the substitution of a fine copper screen for the cloth used to retain the feces. Collections of feces and urine were made daily, and the length of collection periods in all cases, except where otherwise indicated in the tables, was 10 days.

The urine and washings from the crates were aliquoted daily and composited for analysis. The feces were collected, dried at temperatures of 65° C. or less, ground finely, and analyzed. Nitrogen determinations on feed, feces, and urine were made according to the Gunning-Arnold-Dyer modification of the Kjeldahl method.

The pigs were first placed on a nitrogen-free ration consisting of a high-grade cornstarch 96 per cent and a mineral mixture 4 per cent. The mineral mixture used contained steamed bone meal 30 parts, ground limestone 30 parts, sodium chloride 30 parts, magnesium carbonate 3 parts, potassium carbonate 3 parts, potassium sulphate 2 parts, ferric chloride 1.5 parts, and potassium iodide 0.5 part.

After 12 to 14 days on this ration it was found that the output of urinary nitrogen had reached a level, and the first standardizing period was started at this time, using the starch and salts ration.

The second experimental period involved the feeding of corn, the third period the feeding of corn and tankage in the proportion of 2 parts of corn nitrogen to 1 part of tankage nitrogen, the fourth period the feeding of tankage alone, while the fifth period was a final standardizing period on starch and salts. All of these rations contained 4 per cent of the mineral mixture described above and either 2 or, in the later experiments, 5 per cent of cod-liver oil. The amount of protein-containing foods and of starch used was adjusted so that the ration would contain approximately 8 to 10 per cent of crude protein, but this result was not attained in all cases; the nitrogen content of the rations exclusive of the cod-liver oil, which was added when the rations were offered to the pigs, was as shown in Table 1.

TABLE 1.—*Nitrogen content of the experimental rations*<sup>a</sup>

Pig Nos.	Nitrogen content of—		
	Corn	Corn and tankage	Tankage
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
1, 2, 3.....	1.28	1.06	1.25
4, 5, 6, 7, 8.....	1.39	1.49	1.62

<sup>a</sup> These rations were analyzed before the addition of the cod-liver oil. The cod-liver oil was added to the feed just before it was given to the pigs.

A good grade of yellow corn and guaranteed 60 per cent tankage were used in these experiments. The experimental periods were separated by 7 to 10 days of preliminary feeding, in which the pigs received the same rations in the same amounts as were fed in the following collection period.

### EXPERIMENTAL RESULTS

The results of these nitrogen balance studies are assembled in Table 2, together with the biological values computed from them and the more significant intermediate values.

The biological values calculated for the different rations presumably represent the percentages of the absorbed nitrogen used by growing pigs for both maintenance and growth. Their calculation involves the assumption that the excretion of fecal nitrogen per kilogram of food consumed on the nitrogen-free ration in the first and final periods measures the excretion of body nitrogen in the feces in the intervening periods.<sup>3</sup> The change in the excretion of the fecal nitrogen per kilogram of nitrogen-free ration from the first to the fifth period is assumed to occur in a linear fashion with respect to time. The second assumption involved in these calculations is that the excretion of nitrogen in the urine per kilogram of body weight in the first and final periods of nitrogen-free feeding is a measure of the excretion of body nitrogen in the urine in the intervening periods, the change in these values from the first to the fifth period being also assumed to be linear.

For convenience of study, the biological values obtained from the nitrogen metabolism experiments have been collected in Table 3, together with computations of the true digestibility of the dietary protein, allowance being made for the metabolic fecal nitrogen.

<sup>3</sup> In a recent paper appearing in this Journal (16), H. W. Titus concludes that "the fecal nitrogen excretion of a steer consuming a nitrogen-free ration can not safely be taken as a measure of the metabolic nitrogen in the feces of the animal when consuming an equal weight of a given feeding stuff." It appears to the present writers, however, that this conclusion is based upon a debatable mathematical interpretation of data obtained on nitrogen-containing rations. Equation (3) of this article seems contradictory to the conclusion stated, and yet it is used in the argument upon which the conclusion is based. From the fact that the total fecal nitrogen in his experiments appeared to be positively correlated with the moisture content of the feces, Titus concludes that "the amount of metabolic nitrogen in the feces of a steer is influenced, among other things, by the water content of the feces," without disposing of the equally plausible alternative explanation that the digestibility of the feed nitrogen is so influenced. Thus, it is quite possible that equation (2) would be more accurate if it did not contain the *b* term; in which case, again, the conclusion of Titus first quoted would become untenable. Therefore, there appears to be nothing in the data or arguments that Titus has offered which necessarily contradicts the position taken in this and other papers from this laboratory, that the metabolic nitrogen in the feces from a given ration may be satisfactorily estimated from the fecal nitrogen produced on an equal amount of nitrogen-free dry matter of approximately the same crude-fiber content.

TABLE 2.—Nitrogen metabolism data and the calculation of biological values

PERIOD 1. STARCH RATION													
Animal No.	Initial weight	Final weight	Food intake	Nitrogen intake	Fecal nitrogen	Metabolic nitrogen in feces	Food nitrogen in feces	Absorbed nitrogen	Urinary nitrogen	Endogenous nitrogen in urine	Food nitrogen in urine	Food nitrogen retained	Biological value
	Kgm.	Kgm.	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	Pct.
1.....	35.2	35.4	520	.....	0.58	<sup>a</sup> 1.12	.....	.....	1.81	<sup>b</sup> 0.051	.....	.....	.....
2.....	35.2	36.5	520	.....	.43	<sup>a</sup> .83	.....	.....	1.80	<sup>b</sup> .050	.....	.....	.....
3.....	39.0	39.0	520	.....	.45	<sup>a</sup> .87	.....	.....	2.07	<sup>b</sup> .053	.....	.....	.....
4 <sup>c</sup> .....	56.2	52.6	1,000	.....	.72	<sup>a</sup> .72	.....	.....	4.39	<sup>b</sup> .081	.....	.....	.....
5 <sup>c</sup> .....	50.3	50.8	1,000	.....	1.04	<sup>a</sup> 1.04	.....	.....	2.71	<sup>b</sup> .054	.....	.....	.....
6 <sup>d</sup> .....	43.1	45.4	1,000	.....	.54	<sup>a</sup> .54	.....	.....	3.47	<sup>b</sup> .074	.....	.....	.....
7 <sup>e</sup> .....	43.1	43.5	1,000	.....	.75	<sup>a</sup> .75	.....	.....	2.48	<sup>b</sup> .057	.....	.....	.....
8 <sup>e</sup> .....	50.3	49.9	1,000	.....	.90	<sup>a</sup> .90	.....	.....	3.75	<sup>b</sup> .075	.....	.....	.....
PERIOD 2. CORN RATION													
1.....	37.6	37.4	600	7.68	2.16	0.62	1.54	6.14	4.55	1.95	2.60	3.54	58
2.....	37.6	37.4	600	7.68	1.79	.55	1.24	6.44	5.08	1.87	3.21	3.23	50
3.....	40.8	40.6	600	7.68	2.01	.56	1.45	6.23	4.84	2.08	2.76	3.47	56
4.....	57.6	58.1	1,000	13.62	2.51	.77	1.74	11.88	8.82	4.27	4.55	7.33	62
5.....	52.2	54.0	1,000	13.62	3.03	1.00	2.03	11.59	8.85	2.82	6.03	5.56	48
6.....	49.9	51.3	1,000	13.62	2.32	.62	1.70	11.92	8.91	3.31	5.60	6.32	53
7.....	45.8	46.7	1,000	13.62	2.61	.93	1.68	11.94	8.60	2.85	5.75	6.19	52
8.....	51.7	52.6	1,000	13.62	2.76	.88	1.88	11.74	8.85	3.46	5.39	6.35	54
PERIOD 3. CORN AND TANKAGE RATION													
1.....	38.1	38.6	700	7.29	1.72	0.66	1.06	6.23	4.35	2.01	2.34	3.89	62
2.....	38.6	39.0	700	7.29	1.39	.71	.68	6.61	4.35	1.92	2.43	4.18	63
3.....	41.3	43.8	700	7.29	1.63	.69	.94	6.35	4.70	2.08	2.62	3.73	59
4.....	60.1	61.2	1,000	14.60	4.67	.82	3.85	10.75	7.94	4.06	3.88	6.87	54
5.....	56.0	57.4	1,000	14.60	3.97	.96	3.01	11.59	7.81	3.00	4.81	6.78	58
6.....	53.1	55.6	1,000	14.60	2.97	.70	2.27	12.33	7.53	3.09	4.44	7.89	64
7.....	48.5	50.8	1,000	14.60	4.51	1.11	3.40	11.20	7.74	3.28	4.46	6.74	60
8.....	54.2	56.2	1,000	14.60	4.26	.85	3.41	11.19	8.24	3.19	5.05	6.14	55
PERIOD 4. TANKAGE RATION													
1.....	38.6	39.7	700	8.61	1.94	0.60	1.34	7.27	5.81	2.08	3.76	3.51	48
2.....	38.6	39.7	700	8.61	1.87	.76	1.11	7.50	5.88	1.91	3.97	3.53	47
3.....	41.7	41.1	700	8.61	2.21	.74	1.47	7.14	6.90	1.93	4.97	2.17	30
4.....	64.4	65.3	1,000	15.89	5.69	.87	4.82	11.07	9.65	3.88	5.77	5.80	43
5.....	59.4	59.9	893	14.19	5.04	.81	4.23	9.96	8.95	3.13	5.32	4.14	42
6.....	57.2	58.5	1,000	15.89	5.16	.78	4.38	11.51	10.40	2.79	7.61	3.90	34
7.....	53.1	55.3	1,000	15.89	4.84	1.29	3.55	12.34	9.46	3.81	5.65	6.69	34
8.....	56.9	59.2	1,000	15.89	6.62	.82	5.80	10.09	9.70	2.86	6.84	3.25	32
PERIOD 5. STARCH RATION													
1.....	40.1	41.5	700	.....	0.54	<sup>a</sup> 0.77	.....	.....	2.19	<sup>b</sup> 0.054	.....	.....	.....
2.....	39.9	40.8	700	.....	.82	<sup>a</sup> 1.17	.....	.....	1.96	<sup>b</sup> .048	.....	.....	.....
3.....	40.8	41.3	700	.....	.77	<sup>a</sup> 1.10	.....	.....	1.83	<sup>b</sup> .045	.....	.....	.....
4 <sup>c</sup> .....	66.5	69.6	1,000	.....	.92	<sup>a</sup> .92	.....	.....	3.60	<sup>b</sup> .053	.....	.....	.....
5.....	61.7	64.4	1,000	.....	.87	<sup>a</sup> .87	.....	.....	3.29	<sup>b</sup> .052	.....	.....	.....
6.....	59.2	61.2	1,000	.....	.82	<sup>a</sup> .82	.....	.....	2.84	<sup>b</sup> .039	.....	.....	.....
7 <sup>c</sup> .....	54.7	55.6	1,000	.....	1.47	<sup>a</sup> 1.47	.....	.....	4.12	<sup>b</sup> .075	.....	.....	.....
8.....	60.3	61.2	1,000	.....	.80	<sup>a</sup> .80	.....	.....	2.48	<sup>b</sup> .041	.....	.....	.....

<sup>a</sup> Fecal nitrogen per kilogram of dry feed. These values were used in estimating the metabolic nitrogen in the feces in periods 2, 3, and 4. The change in the ratio of metabolic nitrogen to dry matter consumed from period 1 to period 5 was assumed to occur in a linear fashion.

<sup>b</sup> Urinary nitrogen per kilogram of body weight. These values were used in estimating the endogenous (body) nitrogen in the urine in periods 2, 3, and 4, the same assumption of a linear variation from period 1 to period 5 being made as in the case of the metabolic nitrogen in the feces.

<sup>c</sup> This is a 7-day collection period instead of a 10-day period.

<sup>d</sup> This is a 4-day collection period.

<sup>e</sup> This is an 8-day collection period.

TABLE 3.—A summary of the coefficients of digestibility and the biological values of protein

Pig No.	Coefficients of digestibility of protein. <sup>a</sup>			Biological values of protein		
	Corn	Corn and tankage	Tankage	Corn	Corn and tankage	Tankage
1.....	80	85	84	58	62	48
2.....	84	91	87	50	63	47
3.....	81	87	83	56	59	30
4.....	87	74	70	62	64	48
5.....	85	79	70	48	53	42
6.....	87	84	72	53	64	34
7.....	88	78	78	52	60	54
8.....	86	77	63	54	55	32
Average.....	85	82	76	54	61	42

<sup>a</sup> Corrected for metabolic nitrogen in the feces.

The experiments on pigs 1, 2, and 3 preceded the experiments on pigs 4, 5, 6, 7, and 8 by about one year.<sup>4</sup> Some of the differences in the results obtained with these two groups of animals, particularly with reference to digestibility of the protein, may therefore be related to differences in the lots of feed used.

The digestion coefficients were obtained from the estimates of absorbed nitrogen given in Table 2, which involve the assumption that the excretion of total fecal nitrogen in periods 1 and 5, when the pigs were on nitrogen-free rations, measures the excretion of metabolic nitrogen in the feces on equal amounts of protein-containing rations. The justification for this assumption has been discussed at some length by one of the writers in another publication (12).

In these trials the average true digestibility of the nitrogen of corn was 85 per cent. The coefficients for the last five pigs were appreciably higher than those for the first three pigs. The tankage used with the group of five pigs was evidently much less digestible than that used for the group of three pigs. This difference is reflected in the coefficients obtained with the combination of corn and tankage.

The biological values, however, do not show these group differences, although considerable variation among individual results is evident for each ration. The average value for corn protein alone was 54, for tankage protein alone 42, and for corn and tankage proteins in the proportion of 2 to 1, 61. The average results thus confirm the general conclusions of the rat-feeding experiments previously published (11) to the effect that tankage protein (nitrogen) is of lower biological value than the protein of corn, but that, when combined with corn proteins, a protein mixture superior in quality to that of corn is obtained.

Each of the eight pigs, with one exception, gave the lowest biological value for tankage proteins, an intermediate value for corn proteins, and the highest value for the combined proteins. The exceptional pig, No. 7, would fall in line with the others if the exceptionally high biological value for tankage protein could reasonably be considered in error. In looking over the data for this pig for period 4 on tankage

<sup>4</sup> The experiments on pigs 1, 2, and 3 were run from Sept. 24 to Dec. 23, 1925; those on the remaining pigs were run from Nov. 1, 1926, to Feb. 10, 1927.

feeding, the urinary nitrogen is not exceptionally low, but the absorbed nitrogen appears to be estimated too high, due to an abnormally high metabolic nitrogen factor obtained in the following period. The individual data may reasonably be considered, therefore, as substantiating unanimously the conclusion indicated by the average biological values for the proteins of the three experimental rations.

The low biological value of tankage proteins (nitrogen) is not a matter of surprise when it is considered that much of the protein material of which tankage is composed is of low biological value. The bone in tankage, as well as much of the soft tissues, carries albuminoid proteins (particularly collagen) of low nutritive value, and the blood protein, which is commonly added for the purpose of adjusting the nitrogen content to the guaranteed value, must also be classed as of low biological value. Steck (14) has obtained very definite indications that hemoglobin, constituting almost two-thirds of the protein of blood, is poorly utilized in anabolism, while Hoagland and Snider (4) have not obtained economical growth in rats on blood serum proteins alone.

Nor is the marked supplementary relation between tankage and corn proteins quite unexpected. Animal tissue proteins have been shown to possess a high supplemental value for cereal proteins by one of the writers (13) and, more extensively, by Hoagland and Snider (5).

#### SUMMARY

In nitrogen metabolism studies on eight growing pigs, the true digestibility and the biological value of the proteins (nitrogen) of corn and of tankage, as well as of a mixture of these in the ratio of 2 to 1, were determined by methods that have heretofore been applied to rats only.

The average coefficients of digestibility of protein, corrected for the metabolic nitrogen of the feces, were 85 for corn, 76 for tankage, and 82 for the mixture. Apparently different samples of tankage may differ greatly in this respect.

The average biological values for protein (nitrogen) were 54 for corn, 42 for tankage, and 61 for the combination. The individual pigs, with one doubtful exception, gave values showing the same relations as the average values.

The crude protein of tankage evidently is of low nutritive value in covering the nitrogen requirements of growing swine as well as of growing rats. When combined with corn protein, however, a marked supplementary relation is demonstrable; so that the biological value of the mixed proteins is slightly higher than that of corn alone.

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## STUDIES IN NATURAL HYBRIDIZATION OF WHEAT<sup>1</sup>

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### INTRODUCTION

The wheat plant is normally self-fertilized. Numerous instances of natural cross-fertilization, however, have been recorded. These include crosses between wheats of the same or different species, between wheat and rye, and between wheat and *Aegilops*. The amount of such natural crossing has usually been so small in the aggregate that special preventive measures have not been considered necessary in ordinary investigations with the wheat plant.

In the studies of Nilsson-Ehle (8) <sup>2</sup> the highest percentage of natural hybrids found in any wheat variety was 0.9 per cent. At least 2 to 3 per cent of natural crossing for several years at the Minnesota Agricultural Experiment Station is reported by Hayes (3) and Hayes and Garber (4). Garber and Quisenberry (2) report a maximum of 0.5 per cent of  $F_1$  plants in the Mammoth Red variety grown at the West Virginia station.

Natural crossing in partially sterile species hybrids may be more frequent than in ordinary wheat. About 18.5 per cent of the  $F_3$  plants grown by Hayes, Parker, and Kurtzweil (5), in certain families of crosses between *Triticum vulgare* and *T. durum*, were natural hybrids, evidently due to crossing in the  $F_2$ . In such partially sterile hybrids, unfertilized flowers may remain open for several days, thus allowing easy access of foreign pollen.

Natural wheat-rye hybrids have been reported several times. The most frequent occurrence was reported by Meister (7) at the Saratov (Russia) Experimental Station, where about 20 per cent of the plants in certain winter-wheat plots were natural wheat-rye hybrids in 1918. Leighty and Sando <sup>3</sup> found about 18 per cent natural wheat-rye hybrids in a Chinese variety at Arlington Experiment Farm in 1925.

Popova (9) reports finding hundreds of natural  $F_1$  hybrids between wheat and *Aegilops* in 1921 and 1922 near Tashkent, Turkestan, and in 1922 also near Jan Aryk.

Numerous other instances of natural crossing in wheat have been reported, but an exhaustive review will not be made here. Many of the reports are of one or a few hybrid plants that have been found, while in other cases several such plants are recorded.

<sup>1</sup> Received for publication May 26, 1927; issued December, 1927.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 887.

<sup>3</sup> LEIGHTY, C. E., and SANDO, W. J. NATURAL AND ARTIFICIAL HYBRIDS OF A CHINESE WHEAT AND RYE. Jour. Heredity. (In press.)

## HOW HYBRIDS OCCUR AND HOW RECOGNIZED

At blooming time the glumes of the wheat flower usually open and anthesis occurs in such a manner that a portion or at times possibly all of the pollen from the three anthers is shed into the air. This permits the cross-pollination of other flowers through the liberated pollen falling upon them or being carried to them by the wind. Flowers for any reason devoid of pollen are especially subject to cross-fertilization. Any condition, then, that results in a lack of pollen in some flowers may be conducive to natural crossing. Disease infection, the adaptation of the variety, climatic and soil conditions, or other conditions of the environment may influence pollen production and may thus be factors determining the degree of natural crossing.

The detection of natural hybridization is facilitated if it occurs between plants with strongly contrasted characters. It is not apparent when it occurs between plants of a single homogeneous variety. The greater the number of varieties with contrasted characters grown in proximity to one another, then the more likely is natural crossing to be recognized. The grouping in the nursery of varieties blooming at the same time, as is often done to lessen the difficulties of harvesting, facilitates crossing among them.

In wheat hybrids the factors for certain head characters are dominant, or partly so, to their allelomorphs. Thus the factors for absence of awns, red glumes, and pubescent glumes are more or less dominant to the factors for presence of awns, white glumes, and glabrous glumes, respectively. If a variety having one or more of the recessive factors is the female parent in a cross with a variety having one or more of the dominant factors, the hybrid is recognizable in the  $F_1$ . In the reciprocal cross in which the variety having the dominant factor is the female parent, the  $F_1$  would not be recognized as a rule. In a variety of the Fulcaster type, which is awned and has white, glabrous glumes, the three principal recessive head characters are combined. Any plant, then, with one or more of the allelomorphic dominant head characters found in a variety of the Fulcaster type might be suspected of being a natural hybrid. For this reason, natural crossing may appear to occur more frequently in a variety having the recessive characters.

## MATERIAL AND METHODS

The purpose of this paper is to report the results of observations and experiments on natural crossing in wheat at the Arlington Experiment Farm, Rosslyn, Va., near Washington, D. C., during the period 1917 to 1926. The degree of cross-pollination disclosed by these investigations has been higher than that previously reported by others. In fact, sufficient cross-fertilization between varieties has occurred to demonstrate the necessity of special technic in the conduct of certain agronomic or other investigations when purity of plant material is essential. Some information also has been accumulated relating to the causes of natural crossing.

More than 5,000 nursery rows and from 200 to 300 plots usually are grown each year at the Arlington Experiment Farm. These include most of the botanical types of common wheat as well as

representatives of the different wheat species. Many exotic forms unadapted to this locality are included.

The different varieties and strains are usually arranged in the nursery with reference to their time of ripening and habit of growth, in order to facilitate harvesting and equalize competition between adjacent rows. Very diverse morphological types, however, are often grown side by side or end to end. In 1917 and 1918, when some of the work here reported was done, the different strains were grown in single rows 18 feet long and 12 inches apart. About 124 such rows made up a block or series (Fig. 1.) Each block was separated from the next by a 2-foot path or alley. The arrangement of the varieties grown in plots is based largely on taxonomic characters. Some consideration is given to time of ripening, but this is secondary, and adjacent varieties are usually dissimilar in some major head character. This method is followed to prevent accidental mixing in harvesting and threshing. The field plots are one-fortieth of an acre in size (132 feet long by 8.25 feet wide) with an 18-inch alley between plots. Three replications of each variety are usually grown, and the entire plot is harvested for yield determination.

Many of the varieties grown in the nursery are unadapted to eastern wheat culture. Most of these are discarded after being tested from one to three years, but others are continued as breeding stock or for other special purposes. The varieties grown in plots are largely adapted types, though such partially unadapted varieties as Kanred and Nebraska No. 28 may be continued in test for periods of three to five or more years.

#### CLIMATIC AND GROWING CONDITIONS

The relationship between climate and natural crossing in wheat has been suggested by Howard and Howard (6), who believe that a dry, hot climate is conducive to cross-fertilization. The Arlington Experiment Farm has a decidedly humid climate, the 15-year (1912-1926) average annual precipitation being 40.57 inches for the crop year, July to June, inclusive. For the three months of active growth, April, May, and June, the average rainfall for this 15-year period was 11 inches.

The soil type is classed as Keyport silt loam. By the free use of soiling crops of rye and cowpeas, the occasional spreading of manure, and the application of acid phosphate to the grain crops, a high degree of productivity has been developed.

Straw and grain yields are high, and the tillering of the adapted varieties is above the average. The production of several tillers by each plant extends the blooming period of the variety. In most seasons the secondary tillers do not develop fully, and the fertility of these later heads is often poor. The most common disease of wheat is leaf rust (*Puccinia triticea* Erikss.), but this usually develops late in the season and therefore has little effect on the flowering or seed set of the crop. Other diseases, particularly Septoria, scab (*Gibberella saubinetii* (Mont.) Sacc.), and black chaff (*Bacterium translucens undulosum* EFS.), though less prevalent than leaf rust, may possibly exert some influence on the seed set of wheat.

## NATURAL WHEAT HYBRIDS IN 1918

The seed for the 1918 rod rows was handled with unusual care in the 1917 harvest. After 16 feet had been cut from the 18-foot rows for yield determinations, heads typical of the variety in the row were

123	126			152	345	439	554		408	595	656		699	
122	127			153	344	440	553	688	409	594	657	541	700	789
121	128			154	343	441	552	689	410	593	658	540	701	788
120	129	368	ARG	155	342	442	551	690	411	592	659	539	702	787
119	130	367	ARG	156	341	443	550	691	412	591	660	538	703	786
118	131	366	BRG	157	340	444	549	692	413	590	661	537	704	785
117	132	365	ARG	158	339	423	570	671	414	579	662	536	705	784
116	133	364	ARG	159	338	424	569	672	397	596	645	535	706	783
115	134	363	ARG	160	337	425	568	673	398	595	646	534	707	782
				161	336	426	567	674	399	594	647	533	708	781
				162	335	427	566	675	400	593	648	532	709	780
				163	334	428	565	676	401	592	649	531	710	779
112	137	360	ARG	164	333	429	564	677	402	591	650	530	711	778
111	138	359	ARG	165	332	430	563	678	403	590	651	529	712	777
110	139	358	ARG	166	331	431	562	679	404	589	652	528	713	776
109	140	357	ARG	167	330	432	561	680	405	588	653	527	714	775
108	141	356	ARG	168	329	433	560	681	406	587	654	526	715	774
107	142			169	328	434	559	682	407	586	655	525	716	773

FIG. 1.—Diagram showing location and head characters of the nursery rows in 1917 (heavy lines), seed from which produced the natural hybrids selected in 1918, and the head characters of the near-by rows. The rows were 18 feet long and 12 inches apart. Each series was separated from the next by a 2-foot alley

gathered in separate containers from the remaining 2 feet at the ends. These heads were examined again later and threshed in a plant thresher, in which machine mixture is practically eliminated. The seed obtained was sown by hand in the fall of 1917, but the rows were

covered by a wheel hoe, which may occasionally carry a seed across the alley to the opposite row—especially when the soil is damp—or bring over a seed from an adjoining row.

Despite these efforts to maintain the different strains in pure condition, a surprising number of off-type plants were present in certain rows of the 1918 crop. Many of these apparently were  $F_1$  hybrids resulting from natural crosses with strains grown in adjoining or near-by rows the previous year. In order to test this supposition, a head from each of the off-type plants present in the rows of 19 varieties or strains showing impurity in the 1918 crop was harvested and threshed by hand. The kernels from each head were space planted in head rows the following fall, and data on the head characters of each plant were obtained in 1919. These data are given in Table 1.

TABLE 1.—Varieties of wheat grown in 18-foot rows at Arlington Experiment Farm in 1918 in which off-type plants were found, with head characters of the varieties and off-type plants, the probable origin of the off-type plants based on their breeding behavior, and the percentage of natural  $F_1$  hybrids from 1917 seed

Row No. in 1918	Variety	C. I. No.	Head characters of variety <sup>a</sup>	Off-type plants in 1918 rows of the variety		Breeding behavior of off-type plants in 1919 (number of plants with given head characters) <sup>a</sup>								Probable origin of the off-type plants <sup>b</sup>	Number of plants grown in 1919 from 1917 seed remnants		Percentage of F <sub>1</sub> hybrids from 1917 seed
				Number	Head characters <sup>a</sup>	ARG	AWG	BRG	BWG	ARV	AWV	BRV	BWV		Total	Number with head characters like F <sub>1</sub> hybrids in 1918 rows	
119	Fultz-Mediterranean	5353	AWG	3	ARG	36	15	13	3					X120	365	4	1.1
				2	AWV		16				45			X130			
				2	BWG				34					(c)			
				1	ARG	22		4						X121 or 129			
120	Goens	5354	BRG	3	ARG	40	15	12	4					X119	361	11	3.0
				1	ARV	8		3		13		5		X130			
				1	AWG		22							M119			
131	Mediterranean	5364	BRG	3	ARV	8	14	6	2	37	12	14	7	X130	142	7	1.7
				7	BRG			135	49					X131			
				5	AWG		98		25					X118			
132	Stoner	5365	BWG											or 134	176	60	34.1
				15	ARG	200	74	77	25					X365			
				10	AWV		61		19		201		65	X130			
				11	ARG	152	51	48	16					X110			
139	Red Wonder	5373	BWG											or 138	200	14	7.0
				3	AWG		34		14					or 358			
														X137 or 359			
				6	ARG	86	31	29	12					X94			
				3	AWG		54		30					X154			
155	Fulcaster	5389	BWG	2	ARV	9	5	1	1	23	8	10	4	X342	192	20	10.4
				1	BRV			6	0			14	10	X157			
				1	BRG			9	6					X?			

<sup>a</sup> A=awnless, B=bearded or awned, R=red chaffed, W=white chaffed, V=velvet or pubescent, G=glabrous.

<sup>b</sup> X=result of natural cross with row given, M=result of mechanical mixture with row given.

<sup>c</sup> Probably mechanical mixture or possibly segregates of natural cross previous to 1917.

<sup>d</sup> Segregation for chaff color, although evident, could not be accurately determined on account of discoloration of glumes.

TABLE 1.—Varieties of wheat grown in 18-foot rows at Arlington Experiment Farm in 1918 in which off-type plants were found, with head characters of the varieties and off-type plants, the probable origin of the off-type plants based on their breeding behavior, and the percentage of natural  $F_1$  hybrids from 1917 seed—Continued

Row No. in 1918	Variety	C. I. No.	Head characters of variety	Off-type plants in 1918 rows of the variety	Breeding behavior of off-type plants in 1919 (number of plants with given head characters)								Probable origin of the off-type plants	Number of plants grown in 1919 from 1917 seed remnants				
				Number	Head characters	ARG	AWG	BRG	BWG	ARV	AWV	BRV	BWV		Total	Number with head characters like F <sub>1</sub> hybrids in 1918 rows	Percentage of F <sub>1</sub> hybrids from 1917 seed	
136	Stoner	3394	BWG	6	BRV			35				88		<sup>a</sup> X157	158	43	27.2	
				5	ARV	18		10		78		<sup>a</sup> X158						
				14	ARG	167	55	56	21			X90						
				7	AWG		116		35			or 160						
515	Rudy	3512	BWG	2	ARG	30								X161	140	4	2.8	
				6	ARG	92	41	28	11				or 160					
				1	AWG		17		5				X514					
				2	AWV		9		3		35	14	X516					
551	Rudy (Queen of New York)	3493	BWG	7	ARG	86	33	30	7					X726	238	5	2.1	
													X550					
				2	AWG		42		12				or 552					
				1	ARG	18							X443					
568	Penquite	3539	BRV											or 688	237	20	8.4	
													M550					
				2	ARV					36	8	11	2	or 552				
				2	ARV					31	25							
576	Prosperity	3558	AWG	3	ARV					71	29				(e)	221	6	2.7
				2	AWV													
				1	BRV						23							
				2	BWV							52						
583	No. 8	3570	AWG	2	AWV						60				247	2	.8	
				3	ARG	41	13	19	6					X575				
				4	ARG	98	31							X665				
														X417				
594	(Selection)		BRG											or 677	237	15	6.3	
				1	ARG	24	9							X582				
				1	ARG	19	6	6	1					X600				
				2	ARG	48		18						X593				
599	White Chaff	3591	AWG	1	AWV		4				26			X600	233	11	4.7	
													or 642					
				2	ARG	39	13						X598					
				1	ARG	<sup>d</sup> 21		<sup>d</sup> 7					or 394					
701	Fulcaster	3406	BWG	4	AWV						122			M600	154	2	1.3	
													or 642					
				1	AWV		3		1		20	7	X?					
				4	BRG			59	20				X703					
702	Fultz-Mediterranean	3418	AWG	12	AWG		226		63					X540	237	15	6.3	
													or 702					
				22	ARG	464	180	140	47				X700					
													or 788					
710	Millers Pride	3470	BRG											X700	154	2	1.3	
													or 788					
				5	ARV	25		9		76		23	<sup>d</sup> X709					
				10	AWG		227						X711					
711	New Monarch	3477	AWG	3	AWV		18				49			X709	233	11	4.7	
				3	ARG	38	15	13	5				X710					
													or 778					
				5	ARG	90	29						X531					
712	Pride of Genesee	3491	BWV											or 529	150	0	-----	
				1	AWG		15		7				X530					
				2	ARV	14		5		43		12	X713					
													or 529					

<sup>d</sup> Segregation for chaff color, although evident, could not be accurately determined on account of discoloration of glumes.

\* The off-type plants in this variety are possibly the segregates of a cross between the variety Fishhead (AWV), which was grown next to Penquite (BRV) in 1918. The ARV type may have been overlooked in roguing the 1917 seed heads.

In order to obtain accurate data on the percentage of natural crossing that had taken place in 1917, from 240 to 600 kernels from the 1917 seed remnants of each of 16 of the strains showing off-type plants in 1918 also were space planted in the fall of 1918. From 50 to 80 per cent of these produced plants in 1919, and at harvest time descriptive head data on individual plants were obtained. The off types present were compared with those present in the 1918 rod rows. From the breeding behavior of the 1918 off types, as found in 1919, and the result of growing spaced plants from the 1917 remnant seed, the percentage of  $F_1$  hybrid plants produced from the 1917 seed has been calculated, as shown in the last column of Table 1. The off-type plants present in the 1919 rows from 1917 seed remnants were not tested further, but it is assumed that their breeding behavior would have been the same as that of the off-type plants from the 1918 crop tested in 1919.

A total of 248 off-type plants of the crop of 1918 were tested by growing a head from each in 1919. Of these, 214 segregated in such a manner as to indicate that they were  $F_1$  hybrids. Of the remaining 34 plants, 20 were evidently mechanical mixtures from adjoining rows. Ten of these were in row 710 and were apparently mixtures with row 711. The remaining 14 plants, occurring in row 568 (Penquite), have been interpreted as due to a cross occurring previous to 1917, of which the  $F_1$ , or a heterozygous segregate, was inadvertently included among the seed heads collected from the 1917 crop. Such a cross may have occurred in 1916 when the variety Fishhead, which is awnless with white velvet chaff, was grown next to Penquite, which is bearded with red velvet chaff. The  $F_1$  of this cross is semiawned and red chaffed, and a head of this kind may have been overlooked in the 1917 seed heads.

In several cases noted in Table 1, especially in wheats with velvet or pubescent chaff, the color of the chaff was difficult or sometimes impossible of determination as a result of poor development of color, or of discoloration due to weathering or fungous growths.

The number of hybrid plants present in the 1918 rows of the five strains of the Fulcaster type grown in rows 132 (Stoner), 139 (Red Wonder), 155 (Fulcaster), 159 (Stoner), and 701 (Fulcaster) is especially noteworthy. The percentage of probable  $F_1$  hybrids present in the plants from the 1917 seed remnants in four of these strains tested in 1919 ranged from 7 to 34.1 per cent, being higher than in all other varieties with the possible exception of Penquite (row 568). There is no doubt that unusually heavy natural crossing occurred in the strains of the Fulcaster type in 1917, especially in rows 132 and 159, in which the percentages of hybrid plants were 34.1 and 27.2 per cent, respectively. Three recessive characters are combined in the Fulcaster type of wheat—presence of awns, white glumes, and glabrous glumes. Natural crosses with awnless, red-glumed, or velvet-glumed wheats are readily recognized.

All of the 214 segregating off-type plants tested in 1919 behaved as  $F_1$  hybrids, segregation occurring in approximately the expected ratios. If crossing had occurred previous to 1917, segregates of later generations than the  $F_1$  would have been present and some individuals of several groups with different head characters should be homozygous. Such homozygous forms were not present, so it

appears that all of these 214 off types were actually  $F_1$ . It is also evident that the true-breeding plants interpreted as mechanical mixtures were not homozygous segregates of earlier natural crosses. An instance may be cited. Two awnless-red-glabrous (ARG) glumed plants in Stoner, row 159, bred true to head type. These are interpreted as mechanical mixtures with the adjacent row 160 or with row 90 directly across the alley, inasmuch as in a cross of a BWG  $\times$  ARG occurring previous to 1917 it would be expected that true-breeding BRG and AWG forms, as well as ARG and BWG, would be present in the 1918 row. Likewise 3 of the 5 segregating genotypes expected in such a cross were not present, while the 2 that were present are more probably due to natural crossing with neighboring rows in 1917.

It is concluded, then, that the segregating off-type plants were  $F_1$  hybrids and that the true breeding off types are due to mechanical mixtures, with the exceptions noted.

The above-stated conclusions are strengthened by the fact that it has been possible to determine the probable origin of the off-type plants, as shown in Table 1. In nearly every case the hybrid type found would have resulted if crossing had taken place with plants in adjoining rows or in rows not more than 2 or 3 feet away. The characters possessed by the plants in different rows from which off-type selections were made and by those in their neighboring rows, are shown in Figure 1. Nearly every case of natural crossing has been explained as due to cross-pollinations that may have occurred between plants so located that they may have come into actual physical contact with each other at the time of blooming. A few exceptions are found where the probable parents can not be determined. These may have resulted from transportation of pollen from greater distances, but possibly are due to crosses with mixtures in the row or to other undetermined causes.

The wheat crop of 1917 produced the highest average grain yield in any year of the period 1910 to 1925, inclusive. The precipitation during the spring months was below the average, but during the period from May 15 to 31, inclusive, when most of the blooming occurred, the rainfall totaled 1.06 inches.

The season of 1917, though unusually favorable to the general run of wheat varieties, was comparatively unfavorable to the Fulcaster strains. The average yield of the 22 varieties or strains grown in the varietal plot test was 46.2 bushels per acre, while the average yield of the five Fulcaster types was only 37.2 bushels, the different strains ranging in yield from 34.4 to 38.9 bushels. The three Poole selections averaged 51 bushels, the two Purplestraws 49.9 bushels, and the four Mediterraneans 46.4 bushels. In the rod-row nursery the yield of the Fulcasters was comparable. It is probable that the factors resulting in the comparatively poorer yielding performance of the Fulcasters in 1917 were also factors having to do with natural crossing, as no doubt this type did not produce as much pollen with which to pollinate its own flowers as the Pooles or Purplestraws.

The method of selecting seed heads in 1917 may have resulted in a greater percentage of hybrid plants being found in 1918 than if the seed had been taken from the entire row. In selecting heads for seed purposes in 1917 it was necessary in many cases to take those from secondary tillers. The results obtained in 1924 show that a much

greater percentage of natural crossing occurs in the later secondary tillers than in the earlier primary ones. As these seed plants grew at the ends of the rows, the number of secondary tillers would be greater in proportion than occurs on plants not at the ends. It would happen, then, that more secondary heads would be included in the seed lots than the average for the row.

#### NATURAL HYBRIDS IN THE 1921 FIELD PLOTS

The wheat varieties tested at Arlington Experiment Farm for yield in the fortieth-acre plots contain each year many off-type plants which are of hybrid origin. In the crop of 1921 the variety Nebraska No. 28, which is bearded, with white glabrous glumes and yellow straw, contained about 13 per cent of off-type heads, which were semiawned with glabrous white chaff and purple straw. Natural crossing with a wheat of the Purplestraw or Fultz type was thereby indicated, as the 1920 seed plot of Nebraska No. 28 had been carefully rogued by both writers. The 1921 plots of the variety Kanred contained off types of several kinds, but the total percentage of these was lower than in Nebraska No. 28.

Heads were harvested from representatives of the off types present in 1921 in the four varieties Nebraska No. 28, Kanred, Purplestraw, and Bearded Purplestraw. Each head represented one plant, and the seed of these was space-planted in head rows in the fall of 1921. Poor germination and drowning greatly reduced the stand in many rows, particularly in the selections from Nebraska No. 28, of which 4 of the 15 rows contained 4 or less plants each, none being fully bearded. It is probable, however, that the selections were all alike in constitution, so all of them are combined in the data on breeding behavior given in Table 2. Of 71 plants grown from off types selected from Nebraska No. 28, 51 were awnless (or semiawned) and 20 bearded, which is very near the ratio expected in the  $F_2$  of a cross between an awnless and a bearded wheat. It appears that all 15 selections in Nebraska No. 28 were natural hybrids. It is certain that Nebraska No. 28 was largely cross-fertilized in 1920 by an awnless wheat with purple straw, probably the variety Purplestraw, which begins to bloom a few days later than Nebraska No. 28 but before the latter has finished blooming.

From the 53 off-type heads selected in Kanred, a bearded-white-glabrous-chaffed wheat, 22 proved to be hybrids. The types selected were varied in head character. The bearded-white-glabrous types were selected because they showed greater vigor than Kanred and did not have the Kanred beak. Some of these may have been hybrid, but the differentiating characters were not sufficiently pronounced to make it certain. In the progeny of four of these bearded heads, supposedly recessive in type, awnless forms appeared, indicating that natural crossing had occurred in these heads in 1921.

Six of the 27 selections in Purplestraw and 2 of the 6 in Bearded Purplestraw segregated in such a way as to indicate that they were  $F_1$  hybrids. Ten awnless-white-glabrous heads selected in Purplestraw bred true and probably were merely fluctuating variations in the variety. Other pure-breeding selections in Kanred, Purplestraw, and Bearded Purplestraw were probably mechanical mixtures, or possibly in some instances homozygous segregates of crosses occurring previous to 1920.

TABLE 2.—Numbers and characters of off-type heads selected in four varieties of winter wheat at Arlington Experiment Farm in 1921, with their breeding behavior in 1922

Variety	C. I. No.	Head characters of variety <sup>a</sup>	Off-type plants in 1921		Breeding behavior of off-type plants in 1922 (number of plants with given head characters) <sup>a</sup>					
			Number	Head characters <sup>a</sup>	AWG	BWG	AWV	BWV	ARG	BRG
Nebraska No. 28.....	5147	BWG	15	AWG	51	20	-----	-----	-----	-----
			17	AWG	188	66	-----	-----	-----	-----
			2	AWV	9	1	22	8	-----	-----
			1	BRG	-----	4	-----	-----	-----	4
			1	AWV	-----	-----	16	2	-----	-----
			1	ARG	-----	-----	-----	-----	17	5
			16	BWG	-----	-----	-----	-----	-----	-----
Kanred.....	5146	BWG	6	AWG	Pure.	Pure.	-----	-----	-----	-----
			2	BRG	-----	-----	-----	-----	Pure.	Pure.
			1	ARG	-----	-----	-----	-----	-----	-----
			2	AWV	-----	-----	Pure.	-----	-----	-----
			1	BWG	<sup>b</sup> 3	16	-----	-----	-----	-----
			1	BWG	<sup>b</sup> 7	10	-----	-----	-----	-----
			1	BWG	<sup>b</sup> 1	5	-----	-----	-----	-----
			1	BWG	<sup>b</sup> 1	14	-----	-----	-----	-----
			4	AWG	38	10	-----	-----	-----	-----
			2	ARG	5	-----	-----	-----	33	-----
Purple-straw.....	1915	AWG	10	AWG	Pure.	-----	-----	-----	-----	-----
			3	BWG	-----	Pure.	-----	-----	-----	-----
			3	AWV	-----	-----	Pure.	-----	-----	-----
			5	ARG	-----	-----	-----	-----	Pure.	-----
Bearded Purplestraw..	1911	BWG	2	AWG	22	6	-----	-----	-----	-----
			4	AWG	Pure.	-----	-----	-----	-----	-----

<sup>a</sup> A=awnless, B=bearded or awned, G=glabrous chaffed, R=red chaffed, W=white chaffed, V=velvet or pubescent chaffed.

<sup>b</sup> Behavior due to natural crossing in 1921.

### NATURAL CROSSING IN 1921

In addition to the plant selections made in 1921, an experiment was conducted in which the varieties Gladden, Grandprize, Kanred, Brown Fife, and Fulcaster were grown in the order given in single rows 132 feet long and 8 inches apart. Grandprize and Brown Fife are awnless with red pubescent chaff; all the other varieties are bearded with white glabrous chaff. The seedlings were made in the fall of 1920, and at harvest in 1921 from 40 to 50 heads representative of each of the varieties except Gladden were selected and hand threshed. A portion of this seed was sown in spaced rows in the fall of 1921. The 1922 crop from this seed showed no off types in any variety except Kanred, which contained 11, or 2.64 per cent, natural hybrids in the 416 plants matured. It is possible, however, that all of the other three varieties may have contained natural hybrids not evident in  $F_1$ . Descriptions of the off types in Kanred and their  $F_2$  segregation in 1923 are given in Table 3.

In another experiment with Kanred, 25 heads typical of the variety were selected from a plot in 1921. From the seed of these heads 221 plants were grown in 1922, among which 6  $F_1$  hybrids, or 2.71 per cent, were found. The  $F_2$  segregation in 1923 of these 6 plants is shown in Table 3. Practically the same percentage of natural crossing occurred in the field plot of the Kanred variety in 1921 as occurred in the row specially planted for determination of the extent of natural crossing in contiguous rows.

TABLE 3.—Head characters and breeding behavior of natural  $F_1$  hybrids developed from seed of typical heads of Kanred wheat grown in a specially planted row and of natural  $F_1$  hybrids developed from typical heads of Kanred grown in a plot at Arlington Experiment Farm

F <sub>1</sub> natural hybrids in 1922			F <sub>2</sub> segregation in 1923 (number of plants with given head characters) <sup>a</sup>							
Source and number	Per cent	Head characters <sup>a</sup>	AWG	BWG	ARG	BRG	ARV	BRV	AWV	BWV
From rows:										
7.....	1.68	ARV	29	8	50	13	117	50	43	20
4.....	.96	AWG	76	30						
Total.....	2.64									
From a plot:										
1.....	.45	ARG	11	2	12	9				
5.....	2.26	AWG	64	19						
Total.....	2.71									

<sup>a</sup> A=awnless, B=bearded or awned, R=red chaffed, W=white chaffed, G=glabrous chaffed, V=velvet or pubescent chaffed.

#### EXPERIMENTS IN 1924 TO 1926

Six varieties of wheat, two varieties of spelt, one variety of emmer, and *Aegilops ovata* were sown on October 15, 1923, in 52-foot rows 8 inches apart, in the order shown in Table 4. The varieties were chosen and the order of seeding determined with reference to the head characters and the time of blooming of the varieties, as shown in Table 4. Abruzzes rye was sown in 40-foot rows in a block adjoining one end of the rows of these varieties, and Rosen rye was sown similarly in a block adjoining the other end. This experimental plot was isolated from other sowings, except four plots of Alstroum spelt separated from it by a fortieth-acre plot of winter oats. This seeding of spelt was made October 8 and apparently had no effect on the results obtained.

TABLE 4.—Head characters, blooming period, and seeding arrangement of varieties of grains grown at Arlington Experiment Farm in 1924 for studies of natural crossing

Block 1, 40-foot rows		Block 2, 52-foot rows			Block 3, 40-foot rows
Rye variety	Varieties of wheat and other grains	Head characters <sup>a</sup>	Blooming period in 1924	Rye variety	
Abruzzes (12 rows) -----	1. Purplestraw -----	AWG	May 26-June 2 -----	} Rosen (12 rows)	
	2. Nebraska No. 28 -----	BWG	May 22-June 2 -----		
	3. Red Rock -----	BRG	May 31-June 6 -----		
	4. Dawson -----	ARG	June 1-7 -----		
	5. Brown Fife -----	ARV	do -----		
	6. Kanred -----	BWG	do -----		
	7. Alstroum spelt -----	AWG	June 2-9 -----		
	8. White Bearded spelt -----	BWG	June 2-8 -----		
	9. Black Winter emmer -----	BBIV	June 8-12 -----		
	10. Nebraska No. 28 -----	BWG	May 22-June 2 -----		
	11. Purplestraw -----	AWG	May 26-June 2 -----		
	12. Aegilops ovata -----	BWS	May 26-June 3 -----		

<sup>a</sup> A=awnless, B=bearded or awned, G=glabrous chaffed, R=red chaffed, S=glumes covered with short spines, V=velvet or pubescent chaffed, W=white chaffed, Bl=black chaffed.

At heading time the rows were carefully examined for off-type plants, which were removed before flowering. To insure selling 50 heads of Nebraska No. 28, representing as many plants, were bagged before flowering. This variety originated as a selection from a cross of Big Frame and Turkey, and it was thought that perhaps some of the off-type plants often found in it might be occasional segregates of the original cross. At harvest time in 1924, heads were selected from each wheat variety or species and from *Aegilops ovata*. No material was selected from rows 10 and 11, which were duplicates of rows 2 and 1. In the spelts, Black Winter emmer, Kanred, and *Aegilops*, approximately 40 heads were taken at random, but in the remaining five common wheats a first selection of about 40 heads from each variety included only large heads from tall, vigorous, or primary culms, while a second selection of about 40 heads each was confined to shorter, less vigorous, or secondary culms. These two lots are designated "primary" and "secondary," respectively. All heads were carefully examined for trueness to variety or type and then hand threshed. In the fall of 1924 several hundred kernels of each lot were sown in rows with 4-inch spacing between kernels, but drowning of plants reduced the stand in several instances. It was evident from the plants produced from this seed that considerable natural crossing had occurred in 1924 in most of the wheat varieties, though no natural hybrids between wheat and rye were found. Stand notes were taken, and a head from each recognizable off-type plant present in the variety or species was harvested. All off types, except 12 in Kanred, which were unmistakably hybrid, were hand threshed and grown in spaced rows for segregation data.

In those varieties or species in which dominant head characters could easily obscure an  $F_1$  hybrid, all plants showing such characters as increased height or vigor, lighter glume color, or variation in awning as compared with the type of the variety or species were tested by growing seed from a head of the possible off-type plant. Many of these, as shown by later tests, were merely fluctuating variations. In Dawson, 5 from a total of 42 plants, possessing head characters similar to Dawson but differing in vigor or with lighter red glume color, proved to be hybrid. In Brown Fife, 2 of the 49 possible off types with characters similar to Brown Fife were natural hybrids.

The data on segregation given in Table 5 show that all off-type plants possessing a dominant character, such as absence of beards, present in a variety characterized by its recessive allelomorph, such as presence of beards, were  $F_1$  hybrids.

The head characters of the varieties used in the experiment permitted recognition of the pollen parent of the natural cross. One exception occurred in an awnless, red-glumed plant present in Dawson, described in 1925 as a speltoid type. It was distinctly less spelt-like than the natural hybrids which occurred frequently between the spelts and common wheats. This speltoid plant in 1926 produced nine plants classed as speltoid and nine classed as wheat. All 18 of these were red glumed and white kerneled, indicating that either natural crossing between Dawson strains carrying factors for the spelt form or mutation was responsible for this type of inheritance. The Dawson variety was selected for purity to awnlessness, red glumes, and white seeds, and is not necessarily pure for other characters.

TABLE 5.—Varieties or species of *Triticum* and *Aegilops* grown in an isolated plot at Arlington Experiment Farm in 1924 from which plants were selected in 1925 for testing, with head characters of the variety, species, and selected plants, and the pollen parent of hybrid forms, based on the breeding behavior in 1926 of the selected plants

Variety and species	C. I. No.	Head characters of variety <sup>a</sup>	Plants selected for testing		Breeding behavior of selected plants (number of plants with given head characters) <sup>a</sup>												Pollen parent of hybrid forms		
			Num-ber	Head characters <sup>a</sup>	Spelt types						Wheat types								
					ARV	ARG	RRV	BRG	AWV	BVV	AWG	BWG	ARV	ARG	BRV	BRG		AWV	BVV
Nebraska No. 28 (Triticum vulgare).	5147	BWG	62	AWG															599
			9	ARV															1,637
			14	BRG															4
			8	AWG <sup>b</sup>															20
			6	BWG <sup>b</sup>															40
Purplestraw (T. vulgare).	1915	AWG	3	ARG														23	
			3	ARG														48	
			1	BWG <sup>c</sup>															21
			15	ARG															79
			7	ARV															16
Red Rock (T. vulgare).	5976	BRG	4	AWG <sup>b</sup>														1	
			4	AWG <sup>b</sup>														16	
			2	AWG <sup>d</sup>														23	
			2	ARG														63	
			3	ARG														4	
Dawson (T. vulgare).	6161	ARG	1	ARV														0	
			14	ARG <sup>b</sup>														0	
			11	BRG <sup>a</sup>														462	
			6	ARG <sup>b</sup>														1	
			5	ARG <sup>b</sup>														11	
Dawson (T. vulgare).	6161	ARG	5	ARG <sup>b</sup>														28	
			5	ARG <sup>b</sup>														95	
			2	ARV														20	
			37	ARG <sup>d</sup>														844	

<sup>a</sup> A=awnless or half awned, B=bearded or awned, G=glabrous chaffed, R=red chaffed, S=glumes covered with short spines, V=velvet or pubescent chaffed, W=white chaffed, Bl=black chaffed.

<sup>b</sup> Typical hybrid type of *Triticum vulgare* × *T. spelta*.

<sup>c</sup> Segregating for Kanret beak character.

<sup>d</sup> Homozygous selections.

<sup>e</sup> Speltoid type.

TABLE 5.—Varieties or species of *Triticum* and *Aegilops* grown in an isolated plot at Arlington Experiment Farm in 1924 from which plants were selected in 1925 for testing, with head characters of the variety, species, and selected plants, and the pollen parent of hybrid forms, based on the breeding behavior in 1926 of the selected plants—Continued

Variety and species	C. I. No.	Head characters of variety	Plants selected for testing		Breeding behavior of selected plants (number of plants with given head characters)												Pollen parent of hybrid forms					
					Spelt types						Wheat types											
			Number	Head characters	ARV	ARG	BRV	BRG	AWV	BWV	AWG	BWG	ARV	ARG	BRV	BRG		AWV	BWV	AWG	BWG	
Brown Fife (T. vul-gare)	1933	ARV	8	ARV <sup>b</sup>	78	24	23	6	25	9	11	2	29	14	4	0	8	0	3	0	White Bearded.	
			8	ARV <sup>b</sup>	94	38			34	7			31	13			10		5		Alstrom.	
			1	ARV									10	7	8	3					Red Rock.	
			47	ARV <sup>c</sup>									17	5	4	3	4	3	3	0	Kanred.	
Kanred (T. vulgare)	5146	BWG	8	ARV									47	15	11	5	15	4	3	2	Brown Fife.	
			6	BRG												60			23	Red Rock.		
			4	BWG <sup>b</sup>									51						19	White Bearded.		
			4	AWG <sup>b</sup>								43	8		5				10	Alstrom.		
White Bearded (T. spelta).	1724	BWG	2	ARV	11	3	7	4	3	7	1	2	1	2	0	0	3	0	0	0	Brown Fife.	
			3	BWG <sup>d</sup>									69									
			12	AWG <sup>d</sup>																		
			0	BBI V									268									
Alstrom (T. spelta)	1773	AWG																				
Black Winter (T. di-coctum)	2337	BBI V																				
Aegilops ovata		BWS	2	AWS																	Purplestraw.	

<sup>b</sup> Typical hybrid type of *Triticum vulgare* × *T. spelta*.

<sup>d</sup> Homozygous selections.

Purplestraw was the pollen parent of 72 of the natural hybrids listed in Table 5, of which 62 were present in the Nebraska No. 28 variety. Red Rock was the pollen parent in 36 hybrids, Alstroum spelt in 30, Brown Fife in 29, White Bearded spelt in 28, Dawson in 10, and Kanred in 2. Nebraska No. 28, Black Winter emmer, and *Aegilops ovata* did not cross-fertilize any of the varieties or species in 1924. Black Winter emmer and Alstroum spelt were entirely self-fertilized. Seed of *A. ovata* produced two natural hybrids, the result of cross-pollination with Purplestraw, both of which were sterile. White Bearded spelt was self-fertilized with the exception of two natural hybrids with Brown Fife. All six common wheat varieties were cross-fertilized by at least four different pollen parents. Nebraska No. 28 was the pistillate parent to seven different groups of natural hybrids. Only Black Winter emmer and *A. ovata* failed to pollinate Nebraska No. 28.

In Table 6 is shown the frequency of crossing between the varieties of wheat or spelt, expressed as percentages of the plants tested for natural hybrids in 1925, as listed in Tables 5 and 7. From the types of segregates appearing in  $F_2$  it was possible to determine the percentage of the hybrid forms, the parents being restricted to those grown together in the isolated plot in 1924. The tested plants from each variety that were not hybrid were obviously due to selfing within the variety. The percentage of selfing in each variety also appears in Table 6. The high receptivity of Nebraska No. 28 and Kanred to wind-borne pollen and the infrequency of their action as pollinizers are apparent from these results.

TABLE 6.—Percentage frequency of wheat and spelt varieties as reciprocal male and female parents of the natural hybrids listed in Table 5, and the percentage of self-fertilization in each variety

Female parent	Male parent							
	Nebraska No. 28	Purple-straw	Red Rock	Dawson	Brown Fife	Kanred	White Bearded spelt	Alstroum spelt
Nebraska No. 28.....	78.4	13.0	2.9	0.6	1.9	0.2	1.3	1.7
Purplestraw.....	0	97.6	1.1	.2	.5	0	.3	.3
Red Rock.....	0	.4	99.2	.2	.1	0	0	.1
Dawson.....	0	.7	0	97.5	.3	0	.8	.7
Brown Fife.....	0	0	.1	0	97.8	.1	1.0	1.0
Kanred.....	0	0	2.2	.7	3.0	91.1	1.5	1.5
White Bearded spelt.....	0	0	0	0	.5	0	99.5	0
Alstroum spelt.....	0	0	0	0	0	0	0	100

The seeding arrangement of the several forms of wheat, spelt, emmer, and *Aegilops* included in the original isolated plots in the fall of 1923 was determined partly by the time of blooming of the different forms. From the data given in Table 4 it is seen that blooming was continuous from May 22 to June 12 and that the forms in adjoining rows, with the possible exception of Black Winter emmer, coincided more or less in their time of blooming. Presumably, therefore, abundant opportunity for cross-pollination occurred, except in Black Winter emmer, in which no hybrid plants were found.

TABLE 7.—Numbers of plants grown and numbers and percentage of natural  $F_1$  hybrids, as listed in Table 5, produced by seed from primary and secondary heads or heads taken at random in 1924

Variety	C. I. No.	Number of plants grown from seed of—			Number of natural $F_1$ hybrids in plants from seed of—			Percentage of natural $F_1$ hybrids in plants from seed of—		
		Random heads	Primary heads	Secondary heads	Random heads	Primary heads	Secondary heads	Random heads	Primary heads	Secondary heads
Purplestraw.....	1915		804	562		8	25		1.0	4.4
Nebraska No. 28.....	5147		144	332		15	88		10.4	26.5
Do. <sup>a</sup> .....	5147	315			0			0		
Red Rock.....	5976		553	267		3	4		.5	1.5
Dawson.....	6161		520	220		7	12		1.3	5.5
Brown Fife.....	1933		520	286		4	14		.8	4.9
Kanred.....	5146	267			24			9.0		
Alstroum spelt.....	1733	439			0			0		
White Bearded spelt.....	1724	382			2			.5		
Black Winter emmer.....	2337	306			0			0		
Aegilops ovata.....		189			2			1.1		
Total percentage of natural crossing.....									1.5	8.6

<sup>a</sup> Seed from bagged heads.

Crossing was general among the varieties included in this seeding, occurring frequently between varieties separated by several rows as well as between varieties growing side by side. Four crosses were recorded between Purplestraw wheat and Alstroum spelt grown 32 inches apart in rows 11 and 7. From other observations it is known, however, that wheat pollen may be transported and effect cross-fertilization from considerably greater distances than this.

In the harvest of 1924, heads were taken from primary and secondary culms of five varieties of wheat in the isolated plot, but were taken only at random from the other sowings. The numbers of plants grown in 1925 from seed of these different head lots are shown in Table 7, together with the number and percentage of natural  $F_1$  hybrids included in these plants in each lot. The hybrid nature of the plants was determined by their segregation in 1926, as shown in Table 5. The percentages of  $F_1$  hybrids in the plants from seed of secondary heads were from approximately two to six times as large as the percentages in the plants from primary heads. The percentage of natural hybrids in all plants from seed of the primary heads of the five varieties was 1.5 per cent; in all plants from seed of the secondary heads it was 8.6 per cent, or about six times larger than from the primary ones.

More than one-fifth of the plants of Nebraska No. 28 were  $F_1$  hybrids when grown from seed of open-fertilized heads. Among the 315 plants of this variety, however, grown from seed of bagged and therefore self-fertilized heads, no hybrid plant occurred. This indicates that the variety is pure breeding and that the off types that usually occur in it in such large numbers are due to natural crossing and are not occasional segregates of the original cross.

#### DISCUSSION

In these studies on natural hybridization in wheat, during the 10-year period from 1917 to 1926, the percentage of hybrids found in the different varieties under observation varies from zero to

approximately 34 per cent. Although these investigations were designed to establish the fact and the extent of natural crossing rather than the causes, certain indications as to the factors that are responsible for its occurrence have been obtained.

It appears that seasonal conditions play an important rôle in the extent of natural crossing that occurs, but that in these conditions several factors are concerned. Among these factors rainfall and temperature are probably important. In order to study the relation of rainfall and temperature to natural crossing, Table 8 was prepared. In it are given the daily precipitation in inches and the maximum and minimum temperatures in degrees Fahrenheit from May 1 to June 9 in the years 1917, 1920, 1921, and 1924, and the blooming periods of the varieties and species of wheat studied each year. The same varieties were not under observation throughout the entire period, and those that were included for more than one year were grown under somewhat different conditions from year to year.

Extensive natural crossing occurred in 1917, a year when spring rainfall (April, May, and June) was 1.5 inches below the 15-year average (1912-1926), though, as may be seen in Table 8, there were several showers during the latter part of the blooming period. Only 0.15 inch of rain fell in three showers from May 5 to May 26; blooming began May 18. In 1920 the May rainfall was only 1.6 inches as compared with the 15-year average of 3.51 inches, and no rain fell during the blooming period of the varieties observed. In this year about 13 per cent of natural crossing occurred in Nebraska No. 28. In 1924 Nebraska No. 28 bloomed during a period of frequent showers, rain falling on 7 of the 11 days of the blooming period and totaling 2.31 inches. In this year 21.6 per cent natural crossing occurred in a row of Nebraska No. 28 grown near other varieties. In 1921 Kanred was cross-fertilized by near-by varieties to the extent of approximately 2.7 per cent, as shown by the natural hybrids in 1922; in 1924 the cross-fertilization in Kanred amounted to 9.0 per cent. In 1921 the spring rainfall was 2.65 inches above the 15-year average and the May rainfall 2.95 inches above the 15-year average for the month; rainfall totaling 0.26 inch occurred, however, only on the first and last days of the blooming period of the Kanred variety. In 1924 the rainfall in April, May, and June was 15.21 inches, or 4.21 inches above the average, the highest recorded at Arlington in the 15-year period; and the May rainfall of 6.59 inches, or 3.08 inches above average, surpassed all previous years of this period. Kanred bloomed during a comparatively rainless period, rain occurring only on the first and last days and totaling 0.61 of an inch.

Although showers occurred frequently in 1924 during the blooming period of Nebraska No. 28 and infrequently during the blooming period of Kanred, unusually extensive natural crossing took place in both varieties. In 1920, when Nebraska No. 28 bloomed during a rainless period, 13 per cent natural crossing occurred in that variety, while in the rainy period of 1924, 21.6 per cent natural crossing occurred. The relation of rainfall to natural crossing is not apparent from the behavior observed in these and also in other varieties.

TABLE 8.—Daily precipitation (in inches) and maximum and minimum temperatures (in degrees F.) from May 1 to June 9, inclusive, for the years 1917, 1920, 1921, and 1924, at Arlington Experiment Farm

[The limits of the blooming period of the varieties of winter wheat under observation in which natural hybrids occurred are shown by parallel dashes across the three columns pertaining to each year]

Date		1917			1920			1921			1924		
		Pre- cipitation	Temperature		Pre- cipitation	Temperature		Pre- cipitation	Temperature		Pre- cipitation	Temperature	
			Maxi- mum	Mini- mum		Maxi- mum	Mini- mum		Maxi- mum	Mini- mum		Maxi- mum	Mini- mum
		Inches	° F.	° F.	Inches	° F.	° F.	Inches	° F.	° F.	Inches	° F.	° F.
May	1.....	0.22	81	55	0.38	64	47	1.31	52	48	-----	68	53
	2.....		68	45		63	34		62	45	-----	74	39
	3.....		65	46		64	38		57	46	-----	79	44
	4.....	.87	50	44		65	39	.91	52	47	-----	68	43
	5.....		47	44		65	42	.53	50	48	-----	75	37
	6.....	.01	56	43		68	34	.10	58	46	-----	92	47
	7.....		59	43		62	45		74	43	-----	78	59
	8.....	.05	52	47	.41	67	52		75	49	1.23	69	60
	9.....		62	44		72	42		78	51	.40	70	56
	10.....		64	39		82	44		83	47	.02	64	54
	11.....		70	39		85	48		61	56		54	51
	12.....		63	46		58	54	1.90	60	51	1.57	62	48
	13.....		66	47	.76	52	50	.34	78	51	.10	76	52
	14.....		73	49		58	49	.05	76	58		75	52
	15.....		74	53	.05	64	41		78	59	.50	67	47
	16.....		81	43		70	35	.25	63	53	.34	66	51
	17.....		81	56		70	38		74	44		74	45
	18.....		87	48		74	40		79	45	.04	79	53
	19.....		90	55		73	52		80	52		76	53
	20.....		91	55		78	53		80	50		65	57
	21.....		74	61		78	60		85	55	.66	87	51
	22.....		81	57		81	58		89	60	.16	65	48
	23.....	.09	72	62		69	53		90	62		80	43
	24.....		74	45		59	56	.01	61	53		75	63
	25.....		65	46		67	54	.56	75	49	.64	66	47
	26.....		74	45		72	56		76	55	.14	70	45
	27.....	.39	80	58		80	46		71	53	.58	62	56
	28.....	.21	66	60		83	52		85	58	.04	78	58
	29.....	.37	69	57		84	56		83	63		73	57
	30.....		81	49		84	51	.50	76	66	.17	69	53
	31.....		76	55		81	47		82	55		72	44
June	1.....		86	61		87	52		85	53	.58	75	48
	2.....	.04	86	62		90	61		78	62		75	54
	3.....	.34	85	64		88	65		82	52		74	56
	4.....		82	55		60	58		82	61		75	60
	5.....		83	57	1.19	61	55		78	52		78	50
	6.....	.11	85	67	.83	70	52		79	49		86	55
	7.....	1.57	81	65		78	52		72	59	.03	86	60
	8.....		85	60		80	51		80	60		85	63
	9.....		84	60		76	55		82	65	1.48	86	57

When temperature is considered, the relationship with natural crossing again is not apparent. The extreme diurnal temperature ranges (in degrees Fahrenheit) for the blooming periods were 45–91 in 1917, 35–90 in 1920, 44–90 in 1921, and 43–86 in 1924. It is known that very little blooming occurs when the temperature is below 60°, and that usually none at all occurs at temperatures below about 56°. It is not known, of course, when the flowers bloomed which were cross-fertilized; consequently, the temperatures at which crossing occurs or the temperature conditions during the blooming period or preceding it that favor crossing can only be surmised. It is known, however, that during periods of low temperatures and during rainy

periods, possibly but not certainly when temperature is high as well as low, blooming is delayed until favorable weather again occurs, when dehiscence becomes general. Under these conditions, varieties normally blooming several days apart will open and shed their pollen simultaneously, thus allowing natural crossing to occur between varieties that otherwise would not cross.

Howard and Howard (6) state that "natural cross-fertilization is common" in the Punjab, where all wheat is grown under irrigation, but is "exceedingly rare in nonirrigated [humid] tracts." The following explanation is offered:

Under canal irrigation in the Punjab wheat is usually watered twice after sowing, the last watering taking place after the plants are in ear. Often before the last irrigation the supply of water in the soil is so small that the plants partly wilt during the hottest part of the day, the glumes open and the stigmas are exposed to the air. Under such circumstances in the dry hot climates of the Punjab, cross-fertilization is easy, and it is therefore not surprising that it is so frequent.

These conditions certainly would seldom operate at Arlington Experiment Farm in causing natural crossing in wheat. Even in the driest seasons it has not been observed that wilting of wheat plants has occurred at the blooming period. Usually at this time the soil contains sufficient moisture for use of the wheat plant and often there is an oversupply.

The experiments in natural crossing conducted from 1924 to 1926 (Table 5) show that Nebraska No. 28 was cross-pollinated by all varieties or species of *Triticum* except Black Winter emmer, which flowered too late. Nebraska No. 28 was the first variety to bloom (Table 4), and the last blooming was observed in it the day Alstroum spelt was first observed in bloom; yet eight natural  $F_1$  hybrids between Nebraska No. 28 and Alstroum were present in 1925 in the plants from Nebraska No. 28 seed.

The duration of the flowering period of a variety is influenced by weather and by the number of tillers produced by the plants. In bright, hot weather a variety sown at the rate of 6 pecks per acre on fertile soil will complete its blooming in four days; but in 1924 the blooming periods were prolonged, Nebraska No. 28 requiring 11 days. This was due to the frequent rains and cooler weather and made crossing possible between sorts in which it is normally excluded, such as Nebraska No. 28 and the spelts. Unpollinated stigmas may remain receptive to pollen, however, for several days after the time of dehiscence of the anthers, and early and late varieties may occasionally cross-pollinate under such conditions.

The receptivity of the stigmas of emasculated flowers on heads of winter wheat, representing three-year average data, is shown in Table 9. The data show the percentage of seed set in artificial crosses of common wheat varieties in heads from which the two basal and the tip spikelets and all except the two lower flowers of other spikelets had been removed. The technic reduced the duration of the receptivity of the head, but more than 40 per cent of the flowers of a spike were receptive six days after the first flowering of the spike, and 9 per cent were still receptive on the eighth day. However, the pollen of a head with a similar number of flowers would be shed in two or three days. This difference between the availability of pollen and the receptivity of the stigma is probably

a factor in the nonoccurrence of reciprocal natural hybrids observed in some cases.

The two spelts Alstrom and White Bearded were the pollen parents in 58 natural hybrids with wheat, but on the other hand only White Bearded was the pistillate parent in natural hybrids with wheat, and of these only two occurred. Inasmuch as there was no apparent natural crossing between the two spelt varieties, which bloomed at approximately the same date and were grown adjacent to each other, it may be that these spelts are highly self-fertile.

TABLE 9.—*Receptivity of the stigmas of common wheats, at different ages before and after the first dehiscence of the anthers on a head, to pollen of other wheat varieties during the years 1921, 1922, and 1923 at Arlington Experiment Farm*

Period before or after the first dehiscence of anthers	Number of flowers emasculated and pollinated	Percentage of seed set *	Period before or after the first dehiscence of anthers	Number of flowers emasculated and pollinated	Percentage of seed set *
1 day .....	71	3	6 days .....	199	43
0 day .....	174	23	7 days .....	205	10
1 day .....	134	58	8 days .....	181	9
2 days .....	204	53	9 days .....	153	0
3 days .....	211	61	10 days .....	99	0
4 days .....	192	63	11 days .....	121	0
5 days .....	146	42	12 days .....	80	0

\* The seed obtained was grown to detect any possible selfing.

Varietal differences to cross-pollination may be the expression of the environment. Kanred and Nebraska No. 28 hybridize naturally and on a significant scale with other varieties at Arlington. These two varieties are poorly adapted to eastern conditions, even to the extent of reduced pollen production; but grown in regions where they are adapted, a different reaction to natural crossing could be expected. The primary culms of Kanred and Nebraska No. 28 are hardly comparable in vigor and flower development to the secondary tillers of such adapted varieties as Purplestraw and Red Rock. The secondary tillers of five common wheat varieties produced on an average almost six times as many natural hybrid plants as the primary culms. All five varieties showed the same phenomenon. To a small extent the greater constancy of the variety from seed of the primary heads may be due to the abundance of pollen of its own kind which could cross-pollinate any flowers escaping self-pollination. The secondary heads, being later and more irregular in flowering, would not have the same opportunity for cross-pollination with wind-borne pollen of their own kind. However, it is highly probable that self-sterility of the flowers of the secondary spikes is greater than that of the primary ones. Flowers are often observed in which the anthers are poorly developed and either do not contain pollen or fail to dehisce, but it is known that in many cases the egg cells of these flowers are capable of being cross-fertilized. This phenomenon of greater pollen sterility in comparison with egg sterility is commonly experienced in the  $F_1$  and later generations of both the wheat-rye and the *Aegilops*-wheat hybrids.

Kanred and Nebraska No. 28 have both been discarded from the varietal plot experiments at Arlington Experiment Farm, as neither possesses yield merit, nor is it possible in all years to maintain purity to varietal type without roguing to an extent that affects the yield of the plot. Dawson, a white-kerneled wheat, can not be kept pure for seed character without continued selection. Similar difficulty is experienced in maintaining varietal purity in Genesee Giant, Brown Fife, Shepherd, and others, owing to the dominance of certain characters possessed by these varieties. The impurity of the varieties is in part due to mechanical mixtures, but in addition the data accumulated show that natural crossing may be of great importance.

A practical consideration in the conduct of varietal tests is emphasized by these studies of natural crossing in wheat varieties. It has been the practice at Arlington Experiment Farm to grow varieties dissimilar in spike characters adjacent to one another. This permits the separation of bundles accidentally misplaced in harvesting or by windstorms while in the shock. As the plots are usually harvested and threshed in the same order as they are sown, any accidental mixtures in these operations can more easily be detected. In like manner natural crossings are more likely to be evident. When the characters possessed by a variety through their dominance obscure the  $F_1$  natural hybrids that are present, the evidence of natural crossing would not be apparent until the following year when segregation occurred. Roguing would fail to remove the hybrid forms and the segregates approaching the dominant type. Grouping of similar varieties, or of strains of a single type, such as those of Fulcaster or Mediterranean or Poole, together in the field would practically obscure both mechanical mixtures and mixtures resulting from natural crossing. For example, in a field grouping of Poole, China, Shepherd, and Illini Chief, all awnless with glabrous red chaff and purple straw, roguing would remove neither mechanical mixtures nor natural hybrids between any of these varieties. If the natural crossing or mixture was at all extensive, a variety might lose its identity and react in yield and other tests in a manner meaningless so far as varietal significance is concerned.

The necessity of precautions to insure purity of parental types and the selfing of hybrid material for the conduct of genetic investigations has been recognized at Arlington Experiment Farm. This was pointedly brought out in a genetic study of a character transposed by hybridization from rye to a wheat segregate. The segregate exhibits some degree of self-sterility, and the appearance of as high as 15 per cent of hybrid forms in the strain is expected when selfing the previous season has not been practiced. Hayes, Parker, and Kurtzweil (5) give data indicating that 18.5 per cent of natural cross hybridization had occurred in  $F_2$  segregates from crosses of *Triticum dicoccum*  $\times$  *T. vulgare* and *T. vulgare*  $\times$  *T. durum*.

Carman (1) states of the  $F_3$  generation of open-pollinated wheat-rye hybrids:

It is a matter of very great surprise to us that in this plot there is such a variety of heads that if evidence were suddenly placed before us that all of the varieties of wheat in cultivation sprang from accidental crosses between rye and wheat, we should accept it as in harmony with the appearance of these plants.

No doubt the variety of head types was due to the natural back crossing of the  $F_1$  and  $F_2$  generations with different wheat varieties. In the three cases cited there is present a degree of self-sterility favoring natural crossing which would not be expected in crosses within a species. However, varieties may show self-sterility due to poor vegetative development or pollen abortion, especially in the later tillers, thus giving under certain conditions a high percentage of natural crossing.

The segregation data given in Table 5 probably have been affected to some extent by cross-pollination of the  $F_1$  plants. Several undoubted examples were present in the  $F_2$  material, as, for instance, an awnless red pubescent-glumed plant in a segregating population of Nebraska No. 28  $\times$  White Bearded spelt. The awn ratio in the segregating generation of the natural hybrids between the awnless and bearded wheat varieties does not always show a very close fit to the expected. In the segregation of spelt-wheat crosses the awn segregation, however, is 3 to 1. The spelt-wheat natural hybrids consistently produced in the segregating generation a varied assortment of head shapes varying from the linear-fusiform spelt type to a clavate wheat type. The clavate wheat type of head appeared in the crosses of all six wheat varieties with the spelts, though none of the wheat varieties had any inclination toward clavateness, and the appearance of this type of head probably is due to recombination rather than natural crossing of the  $F_1$ . No spelt forms exhibiting any degree of clavateness were present.

Whether or not it is necessary to protect the plants from natural crossing in genetic investigations probably will depend on the character or characters studied and also on the locality where the material is grown. If, however, unusual ratios, transgressive segregation, or unexpected types are obtained, the probability of contamination by foreign pollen should be considered.

It is very evident from the data presented herewith that natural crossing may be prevalent in wheat. The high percentages recorded in some cases are surprising even to the writers who have recorded the data, and will be even more so to those who are accustomed to think of wheat as a self-fertilized plant. But in fact it is quite probable that only a part of the crossing actually occurring has been detected. Crossing between different plants of the same variety and between plants of varieties with similar head characters would not be evident either in the  $F_1$  or in later generations. It is rather disconcerting to the experimenter to find that this condition obtains or may obtain. To find that it is necessary to protect from crossing material believed to be essentially self-fertilized must add to the difficulties of experimentation or result in uncertainty when such protection is not practiced.

In the light of these results the conception of a field of wheat as being made up of strains self-fertilized through innumerable generations may need modification. With crossing here and there between plants of like and unlike strains, and with occasional plants of other varieties occurring as mixtures, a field of wheat is likely to be a heterozygous aggregate rather than a homogeneous entity.

## SUMMARY AND CONCLUSIONS

Natural hybridization in wheat has been studied at Arlington Experiment Farm during the 10-year period 1917 to 1926, inclusive. From the segregation of plants selected in the years 1917, 1920, and 1924 it is known that natural crossing was frequent in these years.

In some varieties only small amounts of crossing were evident; in others varying percentages were found with a maximum of about 34 per cent occurring in a strain of Fulcaster in 1917.

Varietal differences as regards natural crossing are believed to be due often to environmental conditions. Nebraska No. 28 and Kan-red, which are annually cross-fertilized by other varieties, are both poorly adapted to conditions at the Arlington farm.

Extensive natural crossing occurred both in dry and wet blooming periods.

In 1924 approximately six times as much natural crossing occurred in the secondary heads of five common wheat varieties as in the primary heads.

Two spelts grown in a specially arranged sowing in 1924 were almost entirely self-fertilized but were the pollen parents in 58 natural hybrids with common wheat.

Where natural crossing occurs, the field order of the varieties grown in plots should be arranged to favor the expression rather than the obscuring of the crossing.

Precautions to insure selfing may be necessary in the conduct of certain genetic investigations in wheat.

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# THE RELATION OF ATMOSPHERIC HUMIDITY TO THE DETERIORATION OF EVAPORATED APPLES IN STORAGE<sup>1</sup>

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## INTRODUCTION

One of the most serious problems encountered in connection with the drying of fruits and vegetables is that of deterioration in storage. Various fruits and vegetables behave differently in this respect as a result of a variety of causes. The wide differences in chemical composition of the materials, as in water content, nature and amount of carbohydrate and protein, acidity, tannin content, presence or absence of fats and essential oils, and enzyme content, are responsible for some of the diversity. The differences in treatment given the material in preparation for drying and in the conditions during the drying of the various products are also factors productive of differences in the subsequent behavior of dried materials in storage. Since there are no defined standards for moisture content in the various products, except in the case of evaporated apples, various lots of any given product usually vary considerably in moisture content, not only when stored in bulk prior to packing but also in the final package. In consequence such materials show differences in rate and character of the changes occurring in storage when held under uniform conditions. On the other hand, a product prepared under carefully controlled conditions develops wide differences in appearance, market grade, and palatability, as a result of storage under varied conditions.

With any given dried product the storage factors which are of particular importance are humidity, temperature, and the readiness with which oxygen can gain access to the material. In the case of dried fruits the humidity of the storage chamber and the initial moisture content of the material are factors of outstanding importance in the control of deterioration in storage.

## REVIEW OF LITERATURE

Very little work on the deterioration of evaporated fruits and vegetables under controlled conditions is to be found in the literature.

McGillivray (8)<sup>2</sup> reported results of studies on moisture content of evaporated apples extending over a number of years. Commercially packed fruit with a content of moisture in excess of 27 per cent at time of boxing in practically every case showed fermentation within six weeks after packing. Experimental packs of fruit having 27 per cent moisture content were placed in cold storage for 16

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<sup>1</sup> Received for publication Aug. 15, 1927; issued December, 1927.

<sup>2</sup> Reference is made by number (*italic*) to "Literature cited," p. 905.

months. At the end of that time the moisture content had risen to 34.3 per cent and the material was in considerable part decayed. Similar boxes stored in an office room meanwhile lost considerable weight. When opened, restored to the original weight by sprinkling with sterile water, and repacked, such packages invariably underwent fermentation. This result led McGillivray to condemn very strongly the practice of adding water to dry stock in packing as invariably productive of spoilage. His work led to the adoption of 25 per cent as the permissible moisture content of evaporated apples in Canada.

Prescott, Nichols, and Powers (12) conducted investigations of the spoilage of dehydrated vegetables under various conditions of storage. They isolated a large number of species of bacteria and mold fungi, mainly of the commoner soil forms. Storage in very dry air, at either high or low temperatures, resulted in great reduction in the number of bacteria present. At humidities above 70 per cent and temperatures of 20° to 25° C. various vegetables which had been dried to a residual moisture content of 4 per cent absorbed sufficient moisture from the atmosphere during 7 to 10 weeks of storage to permit rapid development of mold colonies. These investigators concluded that evaporated vegetables could be safely stored only in air-tight containers.

Gore and Mangels (5) found that when turnips, onions, spinach, and cabbage were dried without previous cooking and stored in sealed glass jars in the dark at 70° to 90° F., the moisture content of the material had to be reduced to 5 per cent in order to preserve odor and color unchanged for six months. With moisture content above this limit, darkening, loss of distinctive odor, and development of distinctly unpleasant odors occurred.

Nichols (9) states that "active spoilage" (evidently referring to visible growth of molds) in leaf vegetables such as spinach occurs when the moisture content has reached about 20 per cent, and that starchy and sugary vegetables and fruits generally do not show such spoilage until a moisture content of 25 to 30 per cent has been reached. Enzymic darkening occurred when the moisture content was above 10 per cent but was arrested when moisture was reduced to 5 per cent. Nichols, Powers, Gross, and Noel (10) conclude that "to assure best keeping qualities the moisture content of fruits containing much sugar should not exceed 15 to 20 per cent, while that of other fruits and vegetables should not exceed 5 to 10 per cent, the preference in both cases being for the lower percentage." They emphasize the necessity for employing sealable moisture-proof packages for such products.

Cruess, Christie, and Flossfeder (2) and Cruess and Christie (1) state that grapes dried to 23 per cent moisture after sulphuring will keep indefinitely, but that those with higher moisture contents ultimately mold, as do prunes with more than 25 per cent moisture. Lewis, Brown, and Barss (7) state that in their experimental work with prunes they have adopted 17 to 18 per cent as the proper moisture content, but that in some cases it ran as high as 22 per cent without apparent injury to keeping quality.

It is obvious that in some cases the differences in quantities of residual moisture considered safe by different workers are attributable

to differences in climatic conditions under which the experimental work was done, as in the recommendations for prunes just cited. Despite such minor differences, all workers agree that safety in prolonged storage requires drying of all products to somewhat lower moisture contents than are generally employed in commercial practice.

### EXPERIMENTAL WORK

The present work was undertaken for the purpose of studying the effect of humidity of the storage room as a factor in the deterioration of dried fruits. Of the various important dried fruits, evaporated apples probably undergo most rapid decline in appearance and quality when stored for considerable periods, despite the fact that a definite limit of moisture content of 24 per cent is prescribed by Food Inspection Decision 176 (6). Consequently it was determined to study in some detail the relation of humidity to the changes occurring in this product in storage.

### MATERIAL AND METHODS

The fruit employed was grown in the variety orchard of the Office of Horticulture at the Arlington Experiment Farm, Rosslyn, Va. Five varieties—Ben Davis, Delicious, Rome Beauty, Winesap, and Yellow Transparent—were used. These were chosen by reason of their differences in chemical composition and in color and texture of flesh. Four of the five are employed in some quantity in the commercial production of evaporated apples. Yellow Transparent, which is not used in commercial drying, was also used for the reason that it differs considerably in composition from the others. It is a white-fleshed, soft-textured summer variety, high in acid and astringent content, low in sugar and total solids, and has little distinctive flavor. Ben Davis is a coarse-fleshed white variety, rather low in sugar content for a variety of its season, medium in acidity and astringency, and has little distinctive flavor. Delicious is a yellow-fleshed variety of medium sugar content, low in astringency and exceptionally low in acid content, and has a highly distinctive flavor. Rome Beauty has flesh slightly tinged with yellow, a medium sugar content, low acidity, and a fairly pronounced and characteristic flavor. Winesap has a somewhat yellow, fine-grained flesh, high in sugar content, is of medium acidity and astringency, and has a distinctive flavor.

No analyses of the whole fruits were made, but portions of the same lots were pressed for juice making, and analyses of the freshly expressed juices were made. The results of these analyses, which convey an idea of the particular lots of fruit employed, are recorded in Table 1.<sup>3</sup>

<sup>3</sup> The volumetric permanganate method was employed in determining free reducing sugars and total sugars after acid hydrolysis. Acidity was determined by titration with N/10 sodium hydroxide against phenolphthalein, the results being calculated as malic acid. Total and nontannin astringency were determined by the Loewenthal-Procter method. Total solids were determined by drying portions of the fresh juice to moist dryness on a water bath and bringing to constant weight in a vacuum oven at 80° C. Hydrogen-ion concentrations were made by the use of the Bailey hydrogen electrode, and were checked with the quinhydrone electrode.

TABLE 1.—*Chemical composition of freshly expressed juices of apple material used in storage experiments*

Variety	Constituents (per cent)							
	Acid as malic	Reducing sugar	Cane sugar	Total sugar	Total astringency	Tannins	Non-tannins	Hydrogen-ion concentration
Ben Davis.....	0.520	6.32	3.42	9.74	0.924	0.436	0.488	13.44
Delicious.....	.279	9.00	1.10	10.10	.868	.377	.491	12.46
Rome Beauty.....	.360	6.62	3.79	10.41	.817	.204	.613	12.89
Winesap.....	.520	8.40	4.61	13.01	.859	.430	.429	15.07
Yellow Transparent.....	.680	6.84	1.06	7.90	1.942	1.125	.817	9.23

The fruit used was a portion of the tree-run crop of the varieties harvested when commercially picking ripe and was delivered to the laboratory as soon as picked. It was stored in a basement room until it had reached firm eating-ripe condition. A considerable quantity of each variety was then peeled, cored, and sliced by machine, and the sliced fruit was divided into four portions. One lot was immediately placed in the evaporator without further treatment. A second lot was spread on drying trays and heated to 80° C. (176° F.) for 15 minutes by placing in a closed chamber heated by steam jets placed beneath the trays, then transferred to the evaporator. A third lot was dipped into 2 per cent cold sodium chloride solution for 10 to 15 seconds before spreading on the drying trays, and a fourth lot was exposed on trays to the fumes of burning sulphur in a closed chamber for 30 minutes. All were dried together in a tunnel evaporator at a temperature of approximately 60° to 65° C. (140° to 149° F.).

The four treatments described, when applied to fruit of the five varieties employed, yielded material showing a very wide range in appearance and color as it was taken from the evaporator. The material which received no treatment prior to drying showed considerable brown discoloration at the surface of the slices as a result of oxidations during drying. This was much more pronounced in Yellow Transparent than in the other varieties. The untreated lots in all varieties were distinctly superior to the others in flavor, but considerably less attractive in appearance and color. The material treated with sulphur dioxide was the most attractive in appearance, as it showed no browning or other discoloration. The color was a bright light yellow in Ben Davis and Yellow Transparent and a slightly deeper golden yellow in Delicious, Winesap, and Rome Beauty. The flavor of the sulphured material was very good but scarcely equal to that of the untreated material.

The material treated with sodium chloride was much whiter than that receiving any other treatment, but had a peculiar grayish-white tint. Occasional pieces had a very faint pink discoloration, especially noticeable in Yellow Transparent and Winesap. The flavor was distinctly inferior to that of the sulphured material, but the slight saltiness of this fruit made comparison of the two lots somewhat difficult.

The material heated to 80° C. (176° F.) in a current of steam was considerably brighter in color than untreated material, but much

less attractive in appearance than that treated with sulphur fumes or sodium chloride. Many of the slices collapsed during the drying, becoming thin and semitransparent and adhering firmly to the drying trays. The material was distinctly lacking in the richness and fullness of flavor possessed by the untreated material, but was superior in this respect to that which had been dipped in sodium chloride.

All the lots of fruit were allowed to remain in the evaporator until the pieces contained approximately 15 per cent of moisture. The samples used in the storage experiment consisted of 100 gm. each in one series and 200 gm. in another, and were weighed out in a tared dish and immediately transferred to muslin bags and placed in the storage chambers. Three to five identical samples were prepared from each lot of fruit, in order to permit withdrawal of samples for examination and determination of moisture content at intervals during the experiment.

The storage chambers consisted of tall bell jars of 11,000 c. c. capacity, held in inverted position by suitable supports and covered by heavy glass plates made air-tight by stopcock grease. Atmospheric humidities ranging from 88.8 to 8.5 per cent by steps of approximately 10 per cent were obtained by placing in each of the chambers a comparatively large quantity (1,850 c. c.) of sulphuric acid solution of the proper concentration. The series was completed by adding two chambers, one containing calcium oxide, the other distilled water. This gave a series of 10 chambers having relative humidities respectively of 0, 8.5, 18.8, 37.1, 47.7, 58.3, 70.4, 80.5, 88.8, and 100 per cent.

The samples of fruit were loosely packed into small muslin bags, and the bags placed on perforated porcelain plates supported at some distance above the level of the liquid in the chambers. Care was taken to arrange the bags so that free access of air to all the samples was not restricted. The purpose of the experiment was to simulate the conditions prevailing in fruit stored loosely in bulk, and not those in material packed in final containers.

The series of chambers were placed in an insulated room kept at a constant temperature of 25° C. (77° F.) for the first 12 months of storage, after which it was necessary to transfer them to a laboratory room in which the seasonal fluctuations of temperature ranged between 20° and 30° C. (68° and 86° F.). Since it was possible to employ only one controlled temperature, that chosen was made to approximate the maximum temperatures encountered in common storage. Temperature is a factor of considerable importance in determining the rate of deterioration, so that the rate and extent of deterioration here observed would not be expected to hold in experiments carried on at other temperatures.

The material was examined at intervals of one to two weeks during the first two or three months of storage and at longer intervals thereafter. Detailed notes were made at each examination as to any discoverable changes in appearance, color, and market grade of the material, and at intervals of three or four months one of the duplicate samples of each lot was removed and employed for a determination of moisture content after any changes in color, odor, flavor, and palatability had been determined.

## CHANGES IN APPEARANCE AND PALATABILITY

Alteration in appearance was rather promptly apparent in the fruit in the chambers having high humidities. In the chamber with saturated atmosphere a slight brownish discoloration could be observed in the material after 10 days, and this continued to deepen until all the material was uniformly dark brown in color. This change occurred somewhat less rapidly in the material treated with sulphur dioxide than in that receiving other treatments, but at the end of six to eight weeks the sulphured material was indistinguishable from the other lots. Of the five varieties employed, Yellow Transparent showed discoloration earlier than the others and also developed it somewhat more rapidly.

In the chamber with 88.8 per cent humidity the changes were in all respects identical, with the single difference that they appeared a little later and developed somewhat more slowly. As the material from the series of chambers was examined the alteration in color became less and less evident until a point was reached at which no change in color was apparent. After six weeks' storage this point of no change was reached in the chamber having 70.4 per cent relative humidity, but with continuation of the storage period it shifted to a lower humidity, as indicated in Tables 2 and 3.

Concurrently with the changes in color, alterations in the moisture content, odor, and flavor of the material began to appear. Even at the end of 10 days it was apparent that the material in 100, 88.8, and 80.5 per cent humidity was gaining in weight through absorption of moisture, and this became increasingly apparent in these lots at subsequent examinations. At the end of six weeks the development of mold colonies was evident on occasional slices in all but the sulphured lots in the saturated atmosphere, and the odor of incipient fermentation was apparent on opening both the 100 per cent and the 88.8 per cent chambers. A slightly bitter flavor was apparent in the material dried without any treatment and kept in 100 and 88.8 per cent humidity. These changes became less evident in passing down the scale of humidity, the material in the 70.4 per cent chamber having undergone no discoverable change in color, odor, or flavor, and little if any change in moisture content at the end of six weeks.

In the case of the lots stored at humidities less than 70.4 per cent, it was apparent after six weeks that loss of moisture had occurred in quantities proportional to the relative humidities of the various chambers, but the odor, flavor, and color of the material was unchanged.

In the interval between the sixth and sixteenth weeks of storage, the changes occurring may be summarized as consisting of the appearance of some degree of discoloration in the chambers having 70.4, 58.3, and 47.2 per cent humidity, in the order named, followed by the development of a musty, unpleasant odor and, in the case of the material at higher humidities, of a noticeable alteration in taste. In the 100 and 88.8 per cent chambers absorption of moisture and continued growth of molds and yeasts converted the material within the bags to soggy, decaying masses having a strong odor of fermentation.

TABLE 2.—Condition of evaporated Yellow Transparent apples after 112 days' storage at various humidities

Humidity	Treatment	Presence of molds	Taste	Odor or flavor	Color	General appearance	Grade
Saturation	Untreated	Abundant	Bitter, rancid	Musty, fermenting	Dirty brown	Very poor	Unmarketable.
Do	SO <sub>2</sub>	do	do	do	Very brown	do	Do.
Do	NaCl	do	do	do	do	do	Do.
Do	Steam	do	do	do	Dirty brown	do	Do.
88.8 per cent.	Untreated	Small quantity	do	Very musty	Very brown	do	Do.
Do	SO <sub>2</sub>	do	do	do	do	do	Do.
Do	NaCl	do	do	do	do	do	Do.
Do	Steam	do	do	do	do	do	Do.
80.5 per cent.	Untreated	Occasional	Bitter, stale	do	do	do	Do.
Do	SO <sub>2</sub>	do	do	do	do	do	Do.
Do	NaCl	do	do	do	do	do	Do.
Do	Steam	do	do	do	do	do	Do.
70.4 per cent.	Untreated	None	Somewhat stale	Old sugar-barrel odor	do	do	Do.
Do	SO <sub>2</sub>	do	do	do	do	do	Do.
Do	NaCl	do	do	do	do	do	Do.
Do	Steam	do	do	do	do	do	Do.
58.3 per cent.	Untreated	do	do	do	do	do	Low prime.
Do	SO <sub>2</sub>	do	do	do	do	do	Unmarketable.
Do	NaCl	do	do	do	do	do	Slightly marketable.
Do	Steam	do	do	do	do	do	Good prime.
47.2 per cent.	Untreated	do	A little stale	Somewhat abnormal	do	Medium	Prime.
Do	SO <sub>2</sub>	do	do	do	do	do	Do.
Do	NaCl	do	do	do	Light brown	Good	Good prime.
Do	Steam	do	do	do	Grayish brown	Medium	Do.
Do	Untreated	do	do	do	do	Poor	Low prime.
37.1 per cent.	Untreated	do	Fair	Almost normal	do	Medium	Do.
Do	SO <sub>2</sub>	do	do	do	Light brown	Good	Good prime.
Do	NaCl	do	do	do	Grayish brown	Medium	Do.
Do	Steam	do	do	do	do	do	Low prime.
18.8 per cent.	Untreated	do	Fairly good	do	do	Good	Do.
Do	SO <sub>2</sub>	do	do	do	Bright light yellow	Bright light color	Good prime.
Do	NaCl	do	do	do	Grayish pink	Medium	Do.
Do	Steam	do	do	do	Light brown	Fair	Do.
8.5 per cent.	Untreated	do	Good	do	Bright light yellow	Good	Do.
Do	SO <sub>2</sub>	do	Fair	do	Somewhat pink; grayish	Very good	Choices.
Do	NaCl	do	do	do	Somewhat brown	Fair	Prime.
Do	Steam	do	do	do	do	Good	Good prime.
Completely dry	Untreated	do	do	do	Bright light yellow	do	Prime.
Do	SO <sub>2</sub>	do	do	do	Grayish	Fair	Good prime.
Do	NaCl	do	do	do	Light brown	Good	Do.
Do	Steam	do	do	do	do	do	Do.

TABLE 3.—Condition of evaporated Rome Beauty apples after 112 days' storage at various humidities

Humidity	Treatment	Presence of molds	Taste	Odor or flavor	Color	General appearance	Grade
Saturation.....	Untreated.....	Present.....	Stale, acid rancidity.	Very musty.....	Very dark brown.....	Very poor.....	Unmarketable.
Do.....	SO <sub>2</sub> .....	do.....	do.....	do.....	do.....	do.....	Do.
Do.....	NaCl.....	do.....	do.....	do.....	do.....	do.....	Do.
Do.....	Steam.....	do.....	do.....	do.....	do.....	do.....	Do.
88.8 per cent.	Untreated.....	Small amount.....	Very stale.....	do.....	Dark brown.....	do.....	Do.
Do.....	SO <sub>2</sub> .....	do.....	do.....	do.....	do.....	do.....	Do.
Do.....	NaCl.....	do.....	do.....	do.....	do.....	do.....	Do.
Do.....	Steam.....	do.....	do.....	do.....	do.....	do.....	Do.
80.5 per cent.	Untreated.....	Occasional.....	Stale.....	Old sugar-barrel odor.....	Brown.....	Poor.....	Scarcely marketable.
Do.....	SO <sub>2</sub> .....	do.....	do.....	do.....	do.....	do.....	Low prime.
Do.....	NaCl.....	do.....	do.....	do.....	do.....	do.....	Do.
Do.....	Steam.....	do.....	do.....	do.....	do.....	do.....	Do.
70.4 per cent.	Untreated.....	None.....	do.....	do.....	do.....	Medium.....	Scarcely marketable.
Do.....	SO <sub>2</sub> .....	do.....	do.....	do.....	do.....	do.....	Do.
Do.....	NaCl.....	do.....	do.....	do.....	Light brown.....	do.....	Low prime.
Do.....	Steam.....	do.....	do.....	do.....	Faint.....	do.....	Marketable.
58.3 per cent.	Untreated.....	do.....	Somewhat stale.....	Somewhat abnormal.....	do.....	Poor.....	Low prime.
Do.....	SO <sub>2</sub> .....	do.....	do.....	do.....	Light brown.....	Medium.....	Prime.
Do.....	NaCl.....	do.....	do.....	do.....	Pinkish brown.....	Fair.....	Do.
Do.....	Steam.....	do.....	do.....	do.....	Brown.....	Poor.....	Low prime.
47.2 per cent.	Untreated.....	do.....	Fair.....	Fair.....	do.....	Medium.....	Do.
Do.....	SO <sub>2</sub> .....	do.....	Poor.....	do.....	Slightly brown.....	Fair.....	Good prime.
Do.....	NaCl.....	do.....	do.....	do.....	Grayish pink.....	Poor.....	Prime.
Do.....	Steam.....	do.....	do.....	do.....	Somewhat brown.....	do.....	Low prime.
37.1 per cent.	Untreated.....	do.....	Excellent.....	Good.....	do.....	Fair.....	Good choice.
Do.....	SO <sub>2</sub> .....	do.....	do.....	do.....	Light yellow.....	Excellent.....	Prime.
Do.....	NaCl.....	do.....	Fair.....	do.....	Grayish pink.....	Abnormal.....	Prime.
Do.....	Steam.....	do.....	Good.....	do.....	Somewhat brown.....	Poor.....	Do.
18.8 per cent.	Untreated.....	do.....	Excellent.....	Very good.....	do.....	Fair.....	Fancy.
Do.....	SO <sub>2</sub> .....	do.....	Fair.....	do.....	Bright light yellow.....	Excellent.....	Choice.
Do.....	NaCl.....	do.....	Fair.....	do.....	Pinkish.....	Abnormal.....	Do.
Do.....	Steam.....	do.....	Good.....	do.....	Slightly brown.....	Fair.....	Do.
8.5 per cent.	Untreated.....	do.....	Excellent.....	do.....	Somewhat brown.....	Good.....	High prime.
Do.....	SO <sub>2</sub> .....	do.....	Fair.....	do.....	Bright, light yellow.....	Excellent.....	Extra fancy.
Do.....	NaCl.....	do.....	Good.....	do.....	Rose pink.....	Somewhat normal.....	Choice.
Do.....	Steam.....	do.....	Good.....	do.....	Slightly brown.....	Fair.....	Do.
Completely dry	Untreated.....	do.....	Excellent.....	do.....	Somewhat brown.....	Good.....	High prime.
Do.....	SO <sub>2</sub> .....	do.....	do.....	do.....	Bright, light yellow.....	Excellent.....	Extra fancy.
Do.....	NaCl.....	do.....	Fair.....	do.....	Greenish, slightly pink.....	Fair.....	Choice.
Do.....	Steam.....	do.....	Good.....	do.....	Slightly brown.....	do.....	Do.

The condition of the materials in the various chambers at the end of 112 days of storage is stated in summary form for two of the varieties, Yellow Transparent and Rome Beauty, in Tables 2 and 3. The conditions obtaining in the other varieties were so closely identical with those in Rome Beauty that they do not need detailed separate statements, but a few minor differences will be noted in a subsequent paragraph.

In addition to the statements tabulated in Tables 2 and 3 it may be noted that at the end of 112 days of storage all the material stored at humidities of 47.2 per cent or higher had become rather uniformly brown, regardless of the nature of the treatment given prior to drying. Thus the material treated with sulphur dioxide, while not developing discoloration as early as the other lots, at the end of this time had become indistinguishable from the fruit receiving no treatment prior to drying.

At relative humidities lower than 47.2 per cent there was progressive improvement in color with decrease in the atmospheric moisture of the storage chamber in the case of the sulphured material, and at 18 per cent humidity or less there was no change in color. Untreated material was distinctly browned at all humidities above 18 per cent, while that which had been steamed prior to drying was also browned, but to a lesser degree than untreated material. The material dipped in sodium chloride solution was indistinguishable from that receiving other treatments in the case of the lots stored at higher humidities, but in the material held at lower humidities a distinctly pinkish coloration was present. This was not observed except in material treated with sodium chloride, but it was present in all such material stored in relatively dry air. In the case of Yellow Transparent this pink coloration was first observable in the fruit held at 37.1 per cent humidity and was most pronounced in that held at 18.1 per cent. In the other varieties it was detectable in the salt-treated material held at 58.3 per cent as a pinkish tinge along the core line and about the vascular bundles of the slices. In material held at 47.2 per cent humidity all the slices were grayish pink at the surfaces but showed a central zone of normal color when cut across. The pink discoloration was still more pronounced in material held at 37.1 per cent and was present in decreasing degree in the material held at lower percentages of atmospheric moisture. The fruit of the different varieties differed somewhat in that the pink color was more pronounced in those which had whitest flesh.

It would appear probable that this pink discoloration is the result of reaction of the absorbed sodium chloride with some constituent of the apple flesh, probably a flavone or tannin. Whatever the nature of the reaction responsible for the development of the pink color, it is peculiar in that the color is most strongly developed in an atmosphere having 18 to 37 per cent relative humidity and decreases in intensity with either increase or decrease in humidity of the storage room. This type of discoloration has been observed by one of the writers in apples evaporated after dipping in sodium chloride and subsequently stored in a very dry atmosphere, and also in overdried commercial evaporated apples which had been salt dipped prior to drying, but it does not seem to have been mentioned in the literature.

From the results obtained with the use of sulphur dioxide and sodium chloride it may be concluded that these agents are effective in

preventing brown discoloration during the drying process, but that the effect is temporary in character. Neither of these agents prevents a gradual browning of the tissues, which ultimately appears in storage, although sulphur dioxide delayed its appearance somewhat longer than sodium chloride under the conditions of these experiments. This is in general agreement with the results of Overholser and Cruess (11) on the effectiveness of these treatments in preventing discoloration in apple juices. Those authors did not investigate the effect of prolonged storage.

The effects of varying degrees of humidity upon changes in odor and flavor of the fruit were of about the same order as the effects upon alteration of color, as indicated in Tables 2 and 3, but developed more slowly. All the samples held at humidities lower than 80.5 per cent remained normal in odor and taste during the first six weeks of storage. Those at higher humidities developed colonies of molds and were markedly abnormal in odor and taste. At the end of 112 days all samples at humidities higher than 70.5 per cent had developed a distinctly bitter, unpleasant taste and an odor strongly suggestive of barrels in which moist, unrefined sugar had been stored. These abnormalities in odor and flavor were perceptible in samples in which no visible growth of mold or other organisms had occurred, as in some of the lots from the 80.5 and 70.4 per cent chambers. In the lots held at humidities below 70.4 per cent, odor and taste became progressively more nearly normal with decreasing humidity, and at a humidity of 37 per cent or less no detectable change had occurred.

Yellow Transparent differed somewhat markedly from the other varieties in that it showed greater alteration in color, odor, and flavor under any given condition than did the other varieties. In other work it has been observed that most early apples of the low-sugar, high-acid types are much like Yellow Transparent, deteriorating rapidly in storage. This fact is familiar to many operators of commercial evaporators, who base their refusal to employ early maturing varieties upon the impossibility of preserving the appearance and quality of the material for more than very short periods.

The behavior of Ben Davis, Delicious, Winesap, and Rome Beauty with respect to alteration in color, odor, and flavor under the various conditions of storage was so closely identical that details with respect to Rome Beauty (Table 3) are fairly representative of the group. The fall and winter varieties, with their relatively higher sugar content, are much better preserved in storage than is Yellow Transparent. The relatively low sugar content and the high tannin content of the variety last named appear to offer conditions favorable to rapid oxidation and other changes involved in deterioration in spite of its higher total and actual acidity.

That hydrogen-ion concentration may be an important factor in controlling the rate of action of oxidase is indicated by the work of Overholser and Cruess (11), who found that in apple juice darkening on exposure to air was progressively lessened by progressively increasing additions of hydrochloric acid. Falk, McGuire, and Blount (4) also found that in potatoes and certain other vegetables, both fresh and dehydrated, the activity of oxidase and peroxidase was progressively inhibited by increasing acidity of the medium. In the present work deterioration was most rapid and greatest in the variety having highest total acidity and hydrogen-ion concentration. This

may indicate that these changes are not produced by enzymes; if they are, other factors outweigh the effect of acidity.

Certain consistent minor differences in the behavior of the dried product made from the different winter varieties which were noted in the course of the work are of sufficient interest to warrant brief statement. At any given degree of humidity, the material of Delicious was invariably darker in color, more abnormal in odor and flavor, and showed more extensive growth of molds, where molds were present, than did samples of Winesap and Ben Davis given identical treatments and stored under the same conditions. Rome Beauty like Delicious showed much greater change in color under given conditions than did Winesap and Ben Davis, but differed in that odor and flavor were as well preserved as in the latter varieties. At the lower humidities, fruit of Delicious and Rome Beauty which had been salt dipped prior to drying showed distinctly less pink discoloration than did salt-dipped Ben Davis or Winesap from the same storage chambers. The behavior of Rome Beauty differed in one respect from that of all the others, in that the brown discoloration developed at the higher humidities was invariably reddish brown, as contrasted with the light to dark brown, without admixture of red, seen in the other varieties. Much more extended work would be necessary to determine whether these differences are constant for the varieties.

#### FURTHER CHANGES ON EXTENSION OF THE STORAGE PERIOD

As indicated in Tables 2 and 3, the material kept in atmospheric humidities of 100 and 88.8 per cent had been so far altered after 112 days' storage as to be unpalatable and unmarketable. These lots were consequently discarded after samples had been taken for determination of moisture content. The experiment was continued with the remaining chambers, which were held in a room having a constant temperature of 25° C. (77° F.) for one year, then transferred to a room in which the temperatures ranged from 20° to 30° C. (68° to 86° F.) with changes in the season, and there held for a further period of two years and three months. The chambers were opened from time to time for inspection and the making of notes as to condition of the material.

There were no further rapid changes in appearance and color of the material. The lots in atmospheres having humidities of 80.5 to 47.2 per cent underwent a slow, progressive darkening in color, which was most rapid in rate and greatest in degree in the chamber having greatest moisture content and decreased in rate and degree with decrease in humidity of the air present. In the higher humidities there was progressive alteration of odor and flavor, the bitterness and objectionable odor observed earlier in the material stored in approximately saturated atmospheres becoming more and more apparent with the passage of time. These changes were not conditioned upon the development of molds, since they occurred in the absence of any visible growth of organisms. In the chambers having humidities of 37.1 per cent or less the material remained in perfect condition in respect of color, appearance, odor, and flavor, except in the case of Yellow Transparent. In that variety perceptible browning had occurred even in the chamber having 8.5 per cent humidity,

and only the samples held over calcium oxide retained their original color after more than three years.

The experiment was discontinued at the end of three years and three months, when detailed final notes were taken and determinations of moisture content made upon all the lots of material. Minor differences in the several varieties were noted, but these were of the character already described.

#### MOISTURE CONTENT ATTAINED BY THE MATERIAL AT VARIOUS HUMIDITIES

The moisture-absorbing or water-holding capacity of the material at various degrees of humidity is of particular interest. The moisture content of the various lots of material varied slightly when they were placed in the humidity chambers, but closely approximated 15 per cent. As the material gained or lost in weight the sulphuric-acid solutions became more concentrated or more dilute, with a resulting alteration of the humidity of the chamber from its original calculated content. An error was thus introduced, but the quantity of acid used in each chamber was purposely made large enough to reduce the error to a small value, which was further reduced by replacing the acid after the first 8 or 10 weeks of storage.

Moisture determinations were made by weighing out 50 to 100 gm. of the sample and drying to constant weight at 80° C. in a vacuum oven. Table 4 shows the result of the moisture determinations made at the end of the storage period of three years and three months for the samples held in the lower humidities (70.4 per cent or less) and at the end of shorter periods for those in the higher humidities.

The data for the samples in the high humidity chambers (Table 4) are incomplete for two reasons. Many of the samples showed such extensive attack by molds and yeasts that large losses of sugar had occurred. Such samples were weighed to the nearest gram as they were removed from the chambers, no attempt at more accurate determinations being made. Those showing less extensive attack by organisms were sampled for weighing, but some of the samples were destroyed by accident before final weighings were obtained. The crude weighings show sufficiently close agreement with the figures given in the table to make it certain that these figures are truly representative of the condition of the material in these chambers as regards its water content.

TABLE 4.—*Moisture content of evaporated apples of different varieties held in atmospheres of various humidities after attainment of equilibrium* <sup>a</sup>

Humidity	Treatment	Moisture content (per cent)				
		Yellow Trans-parent	Rome Beauty	Winesap	Delicious	Ben Davis
Saturation.....	Untreated.....	54.31	55.46	-----	-----	-----
Do.....	Steam.....	-----	-----	-----	-----	51.74
Do.....	NaCl.....	-----	56.31	-----	-----	-----
Do.....	SO <sub>2</sub> .....	-----	-----	55.23	-----	-----
88.8 per cent.....	Untreated.....	33.35	35.18	-----	36.57	42.04
Do.....	Steam.....	-----	-----	-----	-----	40.38
Do.....	NaCl.....	-----	-----	43.23	44.66	42.00
Do.....	SO <sub>2</sub> .....	-----	-----	-----	46.99	42.25
80.5 per cent.....	Untreated.....	26.56	25.78	25.35	-----	25.63
Do.....	Steam.....	-----	-----	24.54	-----	27.70
Do.....	NaCl.....	-----	27.03	26.76	-----	29.38
Do.....	SO <sub>2</sub> .....	-----	24.93	23.20	25.59	27.36
70.4 per cent.....	Untreated.....	23.42	20.19	19.27	17.65	20.42
Do.....	Steam.....	-----	19.06	19.00	-----	18.66
Do.....	NaCl.....	-----	21.28	21.12	22.21	21.18
Do.....	SO <sub>2</sub> .....	-----	20.59	20.22	21.59	19.73
58.3 per cent.....	Untreated.....	13.69	14.38	13.28	13.74	13.25
Do.....	Steam.....	13.90	13.31	13.00	-----	13.58
Do.....	NaCl.....	14.85	14.81	14.12	13.74	14.75
Do.....	SO <sub>2</sub> .....	13.57	14.24	15.36	12.82	14.27
47.2 per cent.....	Untreated.....	9.79	9.66	9.50	6.62	9.90
Do.....	Steam.....	8.64	8.92	9.00	-----	8.76
Do.....	NaCl.....	10.97	10.21	10.26	11.33	10.70
Do.....	SO <sub>2</sub> .....	8.84	-----	8.47	10.80	10.02
37.1 per cent.....	Untreated.....	7.59	7.67	7.76	6.97	6.20
Do.....	Steam.....	7.00	6.34	6.57	-----	6.51
Do.....	NaCl.....	8.22	7.77	7.45	5.83	7.24
Do.....	SO <sub>2</sub> .....	7.62	7.74	7.00	7.14	6.71
18.8 per cent.....	Untreated.....	4.30	3.87	3.61	2.36	2.95
Do.....	Steam.....	3.78	2.69	3.10	-----	2.02
Do.....	NaCl.....	4.66	4.03	3.85	2.94	2.26
Do.....	SO <sub>2</sub> .....	3.80	4.03	3.56	2.39	2.61
8.5 per cent.....	Untreated.....	2.42	.15	1.97	.61	.89
Do.....	Steam.....	1.57	.11	1.60	-----	.85
Do.....	NaCl.....	2.44	.11	1.80	.33	-----
Do.....	SO <sub>2</sub> .....	1.78	.57	2.01	.32	.63
Completely dry.....	Untreated.....	.40	.53	.47	.74	1.04
Do.....	Steam.....	.41	.16	.63	-----	1.04
Do.....	NaCl.....	.35	.65	.68	.66	.96
Do.....	SO <sub>2</sub> .....	.46	.04	.91	.83	.72

<sup>a</sup> In the case of the lower humidities (70.4 per cent or less) the weighings were made at the end of three years and three months. In the higher humidities the weights recorded are the last it was possible to obtain before invasion by molds proceeded so far as to invalidate the weighings.

Evaporated apples are very highly hygroscopic, and this property is the initial cause for deterioration. When stored in air of any given degree of humidity, the material, regardless of its original moisture content, comes into equilibrium with the atmosphere by gain or loss of moisture. In the present instance material having an initial moisture content of 15 per cent was practically in equilibrium with an atmosphere of 58.3 per cent relative humidity, as almost no gain or loss in moisture content occurred. With increase in humidity there was a rather uniform increase in moisture content of the material, that in a saturated atmosphere taking up somewhat more than its own weight of water. With humidities below 58.3 per cent there was progressive decrease in moisture content of the material, that stored over calcium oxide becoming nearly water free. As the presence of a certain quantity of water in the material is one of the necessary conditions for the occurrence of deterioration, the samples having less than this quantity show no discoverable changes even on greatly prolonged storage, and those having more than this quantity

show a rate and degree of alteration very definitely related to their water content.

The highly hygroscopic character of desiccated plant material has not always been sufficiently considered, or certainly has not always been adequately emphasized, in making recommendations as to "safe" moisture contents for evaporated products. There is no "safe" moisture content for any such material unless it is at once placed under such conditions that absorption of water vapor from the atmosphere can not occur. Unless this is done, drying to any arbitrarily chosen percentage of moisture, no matter how low, can be expected to preserve the material unchanged only so long as the moisture of the surrounding atmosphere does not rise above the point of equilibrium with the product. If this occurs, water will be absorbed to an extent determined by the humidity and the freedom of access of air to the material, and spoilage may occur. This fact is sufficiently important to warrant the placing of decided emphasis upon it in any consideration of preservation of materials of hygroscopic character by drying.

Some conception of the magnitude of the forces concerned in the absorption and retention of moisture by partially desiccated fruits may be gained from consideration of the fact that evaporated apples with 13 to 15 per cent moisture are in equilibrium with an atmosphere of about 58.3 per cent relative humidity, which is obtained over sulphuric acid of 1.30 specific gravity (40 per cent acid). Over acid of 1.25 specific gravity (33.5 per cent acid) such material absorbs an additional 5.7 per cent of moisture, while over acid of 1.35 specific gravity (45 per cent acid) it gives up an approximately equal amount. The assumption that fruit of 15 per cent moisture content is approximately in equilibrium as respects its moisture-absorbing power with sulphuric acid of 1.30 specific gravity thus appears to be justified. While the osmotic pressures of sulphuric acid in high concentrations can not be accurately determined, as Shull (14) has shown, from the use of formulae for "ideal" solutions, calculations by several methods give values ranging from 720 to 798 atmospheres as the osmotic pressure of sulphuric acid of 1.30 specific gravity.

Comparisons of the data on moisture content of material given various treatments prior to drying indicate that such treatments may to some extent affect the water-absorbing capacity of the fruit. That which was dried without treatment and that which had been exposed to sulphur dioxide showed no consistent differences in this respect. That which had been heated to 80° C. in steam rather consistently showed reduced moisture absorption or retention as compared with untreated material, while that dipped in sodium chloride showed somewhat higher moisture absorption, which may be attributed to the salt remaining in and upon the slices.

That the moisture-absorbing capacity of a variety is not directly related to its content is evident from inspection of the data in Tables 1 and 4. Winesap, which is considerably higher in sugar than any of the other varieties, took up smaller percentages of water, especially at the higher humidities, than did the others. Yellow Transparent took up quite as much moisture as any of the others and in a number of cases more, despite its very low content of sugar and total solids. It is evident that the water-absorbing capacity of the several varieties is determined by differences in the character and amount of the

hydrophile colloids present, and that sugar content plays only a minor rôle therein.

A somewhat unexpected outcome of the present work is the discovery that total arrest of deterioration in evaporated apples over prolonged periods can be accomplished only by reducing the moisture content to 8 per cent, as was done in the chamber having 37.1 per cent relative humidity, or a lower figure.

At constant humidities ranging from 47.2 to 80.5 per cent deterioration in color became apparent within a short time and was followed on prolonged storage by development of abnormal, rancid odor and pronounced bitter flavor. At the end of 112 days these changes were well advanced in all the fruit having a moisture content in excess of 18 per cent, and after three years all the material having more than 10 per cent of moisture had become unmarketable if not wholly unfit for consumption. These changes were not accompanied by visible growth of molds or yeasts and occurred under conditions, at least in the lower humidities, which inhibit the growth of these organisms. These changes have been considered as due to the action of enzymes and as being of less importance than those due to molds (9). In the apples here used it is questionable whether enzymes are concerned, since they occur in fruit which has been heated to a temperature of 80° C. (176° F.), or in that treated with sulphur dioxide or sodium chloride, to the same degree as in untreated material.

The work of Overholser and Cruess (11) indicates that although sulphuring or heating to 80° C. does not inactivate the peroxidase of apple tissue, both treatments destroy the organic peroxide and thus inactivate the oxidizing system. That dilute sodium chloride and other alkali chlorides markedly depress the activity of oxidizing systems is indicated by the work of Ewart (3) on the oxidase of apple tissue and by that of Rose, Kraybill, and Rose (13) on that of apple bark. If the discoloration of the tissues is the result of enzyme action, the effects of such agents as heat, sulphur dioxide, and sodium chloride upon the oxidizing system are only temporary in character and are followed by ultimate resumption of activity, notwithstanding the continued presence of sulphur dioxide or salt. Furthermore, the action was most rapid in the case of the variety having highest titratable and actual acidity.

Whatever the cause of the darkening in color and the attendant development of abnormal odors and flavors, these changes are less important than those produced by molds and yeasts only in that they occur more slowly, hence require a longer time to reduce the material to unmarketable condition. But they are extremely important, in that they lower the grade and value of the product in which they occur, and are not arrested by bringing the moisture content of the material to a level at which growth of organisms can not occur.

At moisture contents higher than 25 per cent, the deterioration process develops another phase in consequence of the growth and multiplication of organisms which quickly render the material unmarketable and ultimately wholly destroy it.

Adequate protection against deterioration in evaporated apples can be obtained only by taking into consideration the facts in regard to

the two interrelated but independent types of deterioration here described. Temperature is an important factor in determining the rate and extent of deterioration of both types, but the effects of variation in this factor have not been included in this study.

#### SUMMARY

Evaporated apples of uniform initial moisture content, made from five varieties of rather widely varying types and given four different treatments preparatory to drying, were stored at a temperature of 20° to 30° C. (68° to 86° F.) in atmospheres having humidities ranging from complete saturation to complete dryness for somewhat more than three years, with frequent examination.

The material in all cases either absorbed or gave up moisture until a new moisture content in equilibrium with the moisture of the chamber had been reached.

At relative humidities of 18.8 per cent or less, the material retained its original color, odor, and flavor unchanged throughout the entire storage period. Evaporated apples can be preserved indefinitely by sufficiently reducing the moisture content of both the material and the storage chamber.

At relative humidities between 47 and 80.5 per cent, growth of molds and yeasts did not occur, but the fruit developed progressive brownish discoloration, rancid odor, and abnormal, bitter flavor, which rendered the material unpalatable and unmarketable before the expiration of the storage period. The rate at which these changes occurred increased with increase in humidity throughout the series.

These alterations in color, odor, and flavor became evident within one year in fruit having the moisture content reduced to from 9 to 10 per cent when the material was loosely piled so that air had free access to it. If the changes are produced by enzymes, the enzymes concerned are not destroyed by treatment of the fruit with sulphur dioxide or sodium chloride or by heating it to 80° C. (176° F.) prior to drying.

At relative humidities higher than 80.5 per cent, the absorption of moisture was sufficient to permit rapid and abundant development of molds and yeasts, which destroyed the material within a few months.

Exposure to fumes of sulphur dioxide, immersion in 2 per cent sodium chloride, or heating in steam to 80° C. (176° F.) preparatory to drying, did not prevent the development of discoloration at low humidities or the growth of organisms at the higher humidities, all the lots kept at any given humidity ultimately reaching the condition of the untreated control. Of the various treatments, that with sulphur dioxide retarded the development of these changes somewhat longer than the others. Sodium chloride was next in effectiveness, but its reaction with some constituent of the fruit produced a characteristic alteration of color in some varieties.

The several varieties showed considerable differences in the rate at which deterioration developed, deterioration being most rapid in the variety which combined highest acidity and astringency with lowest content of sugar.

Evaporated apples are highly hygroscopic. Material having 13 to 15 per cent moisture is in approximate equilibrium with the atmosphere over sulphuric acid of 1.30 specific gravity, having an osmotic pressure approximating 750 atmospheres. Fruit having the standard moisture content of 24 per cent is in approximate equilibrium with air of 75 per cent relative humidity at 30° C. (86° F.) and will rapidly absorb moisture when the atmospheric moisture rises above this value.

Adequate protection of evaporated apples against deterioration in prolonged storage involves thorough drying and careful packing of the material and control of the humidity of the storage room. By thorough drying is meant the reduction of contained moisture, not merely to a point at which mold growth can not occur, but to a lower point at which oxidations and other changes not dependent upon the growth of organisms are arrested. Control of humidity in the storage room implies maintenance of atmospheric humidity at or below the point of equilibrium with the material. Careful packing involves the use of the most efficient means of preventing access of atmospheric oxygen to the material.

The temperature of the storage room plays a very significant rôle in determining the rate and extent of deterioration. The present study is not concerned with the effects of storage at various temperatures, all the material having been held at temperatures ranging between 20° and 30° C.

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# DEHULLING BARLEY SEED WITH SULPHURIC ACID TO INDUCE INFECTION WITH COVERED SMUT<sup>1</sup>

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## INTRODUCTION

Since an easy and certain method of obtaining infection is necessary to a successful study of resistance to covered smut of barley, *Ustilago hordei* (Pers.) Kell. and Sw., in hybrid populations of any considerable size, the lack of such a method has delayed investigations to determine the resistance of barley to this disease. To find a simpler and surer method of obtaining high percentages of infection in barley, a series of experiments was begun in the fall of 1925 in the greenhouse of the California Agricultural Experiment Station.

## REVIEW OF LITERATURE

Tisdale<sup>3</sup> obtained a much more abundant infection with covered smut of barley when the seeds were dehulled than when they were left in the natural or hulled state. The hulls were removed by hand with a scalpel or sharp knife, which is at best a slow and tedious process. Seed of Tennessee Winter barley dehulled and inoculated produced 85 per cent of diseased plants, compared with about 4 per cent produced by the inoculated hulled or natural seeds. Similar results were obtained with 10 other varieties. One variety, Nakano Wase, showed no smut even when the seeds were dehulled. Tisdale suggests the desirability of a more efficient method of dehulling the seeds or a simpler means of obtaining satisfactory infection.

Abundant infections with covered smut of barley were obtained by Faris<sup>4</sup> with hulled seed in experiments where the temperature, moisture, and  $P_H$  of the soil were under control during the period of infection. Satisfactory percentages of infection occurred over wide ranges of soil temperature and acidity and at moistures well within those frequently existing when barley is sown in the field. Faris also obtained high percentages of infection in Hannchen and Nepal barleys under ordinary field conditions when the seeds were inoculated with spores collected from these respective varieties. He points out that for these two varieties, at least, the one requirement necessary to produce high infection was to use smut collected from the variety on which it was to be tested.

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<sup>2</sup> Throughout the investigation here reported the writer received many suggestions from W. W. Mackie, Division of Agronomy, University of California, and from various members of the staff of the Office of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, and he wishes to acknowledge the assistance thus rendered.

<sup>3</sup> TISDALE, W. H. AN EFFECTIVE METHOD OF INOCULATING BARLEY WITH COVERED SMUT. *Phytopathology* 13: 551-554. 1923.

<sup>4</sup> FARIS, J. A. FACTORS INFLUENCING INFECTION OF HORDEUM SATIVUM BY USTILAGO HORDEI. *Amer. Jour. Bot.* 11: 189-214, illus. 1924.

The existing knowledge of the resistance of barley to covered smut is comparatively meager and fragmentary. Varietal tests conducted at the California Agricultural Experiment Station by the writer under the immediate direction of Mackie<sup>5</sup> resulted in almost no infection. Other investigators<sup>6</sup> have experienced the same disappointment.

### EXPERIMENTAL METHODS

Experiments in the inoculation of Coast (C. I. 690) barley with covered smut were begun in the fall of 1925. Spores for these experiments were collected from the same variety at Davis, Calif., during the previous summer. Several experiments were tried, in which the seeds were germinated in moist chambers on greenhouse soil, on sand, and on blotter paper moistened with various nutrient solutions. That the spores would germinate readily in the solutions used had been previously determined. In one series, dry spores were dusted on the seeds at the time they were placed in the moist chambers and at daily intervals thereafter until the plumulas were 1 cm. long, when the seedlings were transferred to pots in the greenhouse. In a similar series the seeds were sprayed with a suspension of germinating spores. Practically negative results were obtained in both series. An examination from time to time showed that the spores appeared to be growing normally. Controls of hulled seeds and seeds dehulled by hand were included in each set. The hulled seeds almost invariably produced smut-free plants, whereas the dehulled seeds in soil cultures produced from 20 to 55 per cent of diseased plants. It therefore seemed desirable to look for a simpler method of dehulling the seeds.

The possibility of a chemical method of dehulling the seed was attractive. Sulphuric acid was chosen as a possible reagent because of certain of its properties. It has long been used to treat certain hard seeds in order to promote prompt germination.<sup>6</sup> As early as 1907 Brown<sup>7</sup> found that the seed coat of barley was remarkably resistant to the action of sulphuric acid. He was interested in the semipermeability of membranes, and used a blue-seeded barley for his experiments because the pigment in the aleurone layer is turned red by acids. When the seed coat was intact, soaking in an acid solution of 36 per cent concentration for 44 hours caused no penetration of the seed coat.

### EXPERIMENTS WITH CONCENTRATED SULPHURIC ACID

Preliminary experiments by the writer indicated that barley seeds may germinate after being immersed in concentrated sulphuric acid for one hour. However, the young seedlings produced from seeds treated for 20 minutes showed signs of injury. The seeds usually were immersed in a quantity of acid from two to three times the volume of the seeds. The excess acid was drained off before washing by pouring

<sup>5</sup> STAKMAN, E. C. DISEASES OF CEREAL AND FORAGE CROPS IN THE UNITED STATES IN 1921. U. S. Dept. Agr., Bur. Plant Indus. Plant Disease Bul., sup. 21: 208-209, illus. 1922. [Mimeographed.]

<sup>6</sup> LOVE, H. H., and LEIGHTY, C. E. GERMINATION OF SEED AS AFFECTED BY SULFURIC ACID TREATMENT. N. Y. Cornell Agr. Expt. Sta. Bul. 312, p. 293-336, illus. 1912.

<sup>7</sup> BROWN, A. J. ON THE EXISTENCE OF A SEMI-PERMEABLE MEMBRANE ENCLOSING THE SEEDS OF SOME OF THE GRAMINEAE. Ann. Bot. [London] 21: 79-87. 1907.

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the seeds into a small wire basket which had been coated with paraffin to resist the action of the acid. The basket of treated seeds was plunged into a sink full of cold water and then placed for 10 minutes under a faucet fitted with a rose or shower nozzle. Finally, the excess water was removed with a towel, and the seeds were spread out in the laboratory to dry. Commercial sulphuric acid proved to be nearly as effective in removing the barley hull as C. P. acid and is considerably cheaper. The commercial acid therefore was used in all of these experiments.

Hand-threshed seed of Tennessee Winter (C. I. 257) barley was used for the acid experiments. The smut was from Coast (C. I. 690), as in the former experiment. The seeds were thoroughly dusted with an excess of spores. Twenty-one seeds from each treatment were placed in 8-inch pots and covered uniformly to a depth of 1 inch. The pots were left in the head house, where the temperature was favorable for infection. The temperature ranged from 7.8° to 17.7° C. When the seedlings were 2 or 3 inches high the pots were moved into the greenhouse. The percentages of infection obtained after dehulling barley seeds with concentrated sulphuric acid are reported in Table 1.

TABLE 1.—*Infection with covered smut in Tennessee Winter barley grown from seeds treated with concentrated sulphuric acid and seeded in the greenhouse on February 15, 1926, at Berkeley, Calif.*

[TWENTY-ONE SEEDS USED FOR EACH TREATMENT]

Treatment	Plants produced		Plants smutted	
	Number	Per cent	Number	Per cent
None.....	21	100.0	0	0
Seeds dehulled by hand.....	19	90.5	4	21.1
Seeds treated with concentrated H <sub>2</sub> SO <sub>4</sub> 10 minutes.....	19	90.5	4	21.1
Seeds treated with concentrated H <sub>2</sub> SO <sub>4</sub> 20 minutes.....	5	23.8	2	40.0
Seeds treated with concentrated H <sub>2</sub> SO <sub>4</sub> 30 minutes.....	1	4.8	1	100.0

There was no smut on any plants produced by hulled seeds, whereas 21 per cent of the plants produced by dehulled seeds were smutted. The 10-minute acid treatment resulted in a satisfactory stand of plants which showed the same percentage smutted as the stand from the dehulled seeds. A very thin hull remained after this treatment. It was so thin that the plumules broke through the hull instead of pushing out between the hull and the kernel, as is the rule in hulled seeds. Treatment with acid for 20 or 30 minutes reduced the stand too much to be of practical value.

## EXPERIMENTS WITH DILUTED SULPHURIC ACID

### FIRST EXPERIMENT

Another series of experiments was started with seeds treated with diluted sulphuric acid. A concentration of acid was sought which would digest the entire hull without injury to the seeds. A scale of acid concentrations ranging from 40 to 90 per cent was employed. The time of treatment was somewhat arbitrary in each case, except that a few seeds were removed from time to time and the hulls and

seed coats examined. Forty-five seeds from each treatment were placed in 12-inch pots and covered uniformly to a depth of 1 inch. The pots again were left in the head house until the plants had emerged. The temperature ranged from 10° to 23.3° C. The percentages of smut from the lots of seeds treated with the various dilutions of acid are recorded in Table 2.

TABLE 2.—*Infection with covered smut in Tennessee Winter barley grown from seed treated with various concentrations of sulphuric acid and seeded in the greenhouse on March 11, 1926, at Berkeley, Calif.*

[FORTY-FIVE SEEDS USED FOR EACH TREATMENT]

Lot No.	Con- cen- tra- tion of acid	Dura- tion of treat- ment	Degree of dehulling	Plants produced		Plants smutted	
				Num- ber	Per cent	Num- ber	Per cent
1	40	P. ct.					
2		H. m.					
3							
			Thin hull remaining over entire seed.....	38	84.4	8	21.1
			Thin hull remaining over germ half of seed.....	39	86.7	10	25.6
			Thin hull remaining over germ.....	43	95.6	18	41.9
4	50		Thin hull remaining over germ half of seed.....	39	86.7	17	43.6
5			Thin hull remaining over germ.....	40	88.9	15	37.5
6			Mostly dehulled; thin hull remaining over germ of a few.....	35	77.8	4	11.4
7	60		Thin hull remaining intact.....	45	100.0	6	13.3
8			Thin hull remaining over germ.....	37	82.2	5	13.5
9			Mostly dehulled.....	21	46.7	4	19.0
10	70		Thin hull remaining over germ half of seed.....	43	95.6	7	16.3
11			Mostly dehulled.....	33	73.3	9	27.3
12			do.....	12	26.7	6	50.0
13	80		Very thin hull remaining over entire seed.....	42	93.3	12	28.6
14			Thin hull remaining over germ.....	26	57.8	11	42.3
15			Mostly dehulled.....	3	6.7	1	33.3
16	90		Thin hull remaining over entire seed.....	41	91.1	11	26.8
17			Very thin hull remaining on most.....	30	66.7	14	46.6
18			Thin hull remaining over germ.....	5	11.1	4	80.0
19	Control.		Dehulled by hand.....	41	91.1	11	26.8
20	do.		Hulled.....	42	93.3	0	0

Obviously it is difficult to describe the degree of dehulling in a few words. Three general conditions of dehulling existed, with all the intermediate stages present. After some of the shorter treatments a thin hull still persisted over the entire kernel, a condition similar to that described after the 10-minute concentrated-acid treatment. After treatments of intermediate severity, the hull remained only over the germ. The hull is considerably thicker over the germ and naturally more time is required for the acid to digest this portion. After the more severe treatments the hull was found to be entirely removed from most of the seeds by the acid. Concentration of 80 and 90 per cent acid left the seed coats with a corroded appearance.

Stands of less than 30 plants are not considered desirable and may be dropped from further consideration. Plants from 8 of the 13 remaining treatments contained percentages of smut as high as or higher than those from the seed dehulled by hand. Plants from the hulled lot, on the other hand, were free from smut. The dilute solutions of acid appear to be more desirable than the concentrated solutions, both because of abundant smut infection and because of

lack of seed injury. Just why the 60 per cent acid treatment was followed by such uniformly low percentage of smut can not be explained at this time. An examination of some of the seeds from this treatment indicated that the acid was not as completely removed as after most of the other treatments. The degree of dehulling was as complete as was the case with several other treatments where higher infection occurred. There seems to be considerable variation in smut infection after apparently equal degrees of dehulling. Considerable variation may be expected, however, where such small numbers of plants are used.

TABLE 3.—*Infection with covered smut in Tennessee Winter barley grown from seed treated with various concentrations of sulphuric acid and seeded in the greenhouse on November 26, 1926, at Berkeley, Calif.*

[FORTY-FIVE SEEDS USED FOR EACH TREATMENT]

Lot No.	Concentration of acid	Duration of treatment	Degree of dehulling	Plants produced		Plants smutted	
				Number	Per cent	Number	Per cent
1	P. ct. 30	H. m. 24 0	All dehulled.....	14	31.1	14	100.0
2	40	6 0	Hull remaining on few germ ends.....	33	73.3	29	87.9
3		7 0	All dehulled.....	27	60.0	24	88.9
4		8 0	.....do.....	29	64.4	22	75.9
5	50	4 0	.....do.....	26	57.8	23	88.5
6		5 0	.....do.....	16	35.6	13	81.2
7		6 0	.....do.....	10	22.2	10	100.0
8	60	1 30	Hull remaining over germ end.....	34	75.6	30	88.2
9		2 0	Hull remaining over germ end of half of seeds.....	16	35.6	14	87.5
10		2 30	All dehulled.....	2	4.4	2	100.0
11	70	30	Hull remaining over germ half of kernel.....	40	88.9	29	72.5
12		40	Hull remaining over germ end.....	35	77.8	31	88.6
13		50	Very thin hull remaining over germ end.....	22	48.9	20	90.9
14	80	25	Hull remaining over germ end.....	22	48.9	16	72.7
15		35	Thin hull remaining over germ end.....	3	6.7	3	100.0
16		45	Most kernels dehulled.....	1	2.2	1	100.0
17	90	15	Very thin hull remaining.....	10	22.2	7	70.0
18		20	Hull remaining over germ end.....	10	22.2	7	70.0
19		25	Very thin hull remaining over germ end.....	5	11.1	4	80.0
20	Concentrated	5	Thin hull remaining intact.....	38	84.4	33	86.9
21		10	Very thin hull remaining intact.....	25	55.6	19	76.0
22		15	Hull remaining over germ end.....	3	6.7	3	100.0
23	Control	-----	Dehulled by hand.....	22	48.9	16	72.7
24	do	-----	Hulled.....	45	100.0	12	26.7

#### SECOND EXPERIMENT

On November 26, 1926, another series of experiments was started in the greenhouse, in which were used dilutions of sulphuric acid ranging from 30 per cent to concentrated. Seed of Tennessee Winter (C. I. 257) barley was again employed. Instead of using smut from a single variety, as in the former experiment, the inoculum consisted of a composite sample from the following varieties: Tennessee Winter (Calif. 2272 and C. I. 257), Coast (C. I. 690), Atlas, Club Mariout, Smyrna, Hero Selection (C. I. 1286-1), Vaughn, and Flynn. The seeds were washed for 20 minutes instead of 10, as in previous experi-

ments. Forty-five seeds from each treatment were placed in 12-inch pots and covered uniformly to a depth of 1 inch. During the period of infection the pots were left in the head house, where the temperature ranged from 12.2° to 16.7° C. When the seedlings were about 2 inches high the pots were moved into the greenhouse. The results from this experiment are given in Table 3.

The percentages of smut are much higher after all of these treatments than after those of the preceding experiment. Whether the higher infections were due to more favorable environmental conditions or to the inoculum used is not at present known. The soil and the soil moisture were the same. The temperature in the earlier experiment ranged from 10° to 23.3° C. as compared with 12.2° to 16.7° in the later experiment. The lower maximum temperatures were due to the prevalence of cloudy weather.

The number of plants developing after most treatments was considerably smaller in the later than in the earlier experiment. Even from the lot of hand-dehulled seeds there were only 22 plants in the second experiment as compared with 41 in the first. It is interesting to note that in the second experiment there were 45 plants, or a 100 per cent stand, where the seeds were not dehulled. If 30 or more plants are considered a satisfactory stand, five of the acid treatments were followed by satisfactory stands while hand-dehulled seed was not. Eleven of the acid treatments were followed by stands equal to or better than that from the hand-dehulled seeds. The percentage of smut in the plants from the acid-treated seeds was as high as or higher than in those from the hand-dehulled seeds. The plants from the hulled seeds had a slightly higher percentage of smut in this experiment than those from the hand-dehulled seeds in the previous experiment (Table 2). However, the dehulled seeds allowed much better infections in each experiment than did the hulled seeds.

The treatments with diluted acid caused more complete dehulling in the first experiment than in the second. The reason for this is not apparent at present. The data thus far indicate that it is not necessary to dehull the seed completely in order to induce satisfactory smut infections. In fact, the best stands, accompanied with satisfactory smut infection, resulted where a thin hull remained over the germ end of the seed or over the entire kernel.

#### FIELD EXPERIMENT WITH ACID-TREATED SEED

A very limited experiment with acid-treated seed was made in the field at Davis, Calif., to determine the effectiveness of this method of infection under field conditions. Coast (C. I. 690) barley, inoculated with spores of covered smut from the same variety, was used in this experiment. Duplicate rod rows from each treatment were seeded. Seventy-five seeds were sown in each row and covered as uniformly as possible. The field results are recorded in Table 4.

The plants from the hulled seeds produced 10.3 per cent of smut, which is only about half that produced by those receiving the 50 per cent acid treatment. Seeds treated with concentrated acid for 10 minutes produced results equal to those from the hand-dehulled seeds. Row 8 may have been at a slight disadvantage, as it was located very near an irrigation levee. The acid-treated seeds at least compare favorably with the hand-dehulled seeds both from the standpoint of stand and of smut produced.

TABLE 4.—*Infection with covered smut in Coast barley grown from seeds treated with sulphuric acid and seeded in the field on March 1, 1926, at Davis, Calif.*

[SEVENTY-FIVE SEEDS IN EACH ROW]

Row No.	Treatment	Plants produced		Plants smutted		Average percentage of smut
		Number	Per cent	Number	Per cent	
1	None.....	65	86.7	6	9.3	-----
2	do.....	62	82.7	7	11.3	10.3
3	Dehulled by hand.....	60	80.0	15	25.0	-----
4	do.....	40	53.3	14	35.0	30.0
5	Concentrated H <sub>2</sub> SO <sub>4</sub> , 10 minutes.....	53	70.7	20	37.7	-----
6	do.....	67	89.3	18	27.0	32.3
7	50 per cent H <sub>2</sub> SO <sub>4</sub> , 10 minutes.....	55	73.3	13	23.6	-----
8	do.....	35	46.7	7	20.0	21.8

## DISCUSSION

Hulled seeds produced plants showing only low infections with covered smut, as has been the case in previous experiments at this station. In 1919–20 W. W. Mackie<sup>8</sup> and the writer made duplicate rod-row seedings of 570 varieties and strains of barley at both Davis and Salinas, Calif. Although the seeds were heavily inoculated with viable spores of covered smut, practically no smut occurred. The following year about 100 varieties were sown at each of these stations with similar results. In no case did the smut infection exceed 5 per cent, although from 10 to 15 per cent of smut occurred in a field seeded by a farmer with untreated seed on the same day on land adjacent to one of the stations.

Dehulled seeds constantly produced fairly satisfactory infections. Only in the last experiment were the amounts of smut of the same magnitude as those obtained by Tisdale.<sup>9</sup> As already pointed out, Faris<sup>10</sup> was able to get a very high percentage of infection with hulled seed where the temperature, moisture, and P<sub>H</sub> were controlled. However, these factors are not easily controlled where extensive sowings are necessary. Unsatisfactory infections (Table 4) occurred with hulled seed even when smut from the variety suggested by Faris<sup>11</sup> was used. On the other hand the writer obtained fairly satisfactory infections by dehulling the seeds even under conditions where no smut was produced when hulled seeds were used. Under the conditions of these experiments, plants grown from seeds treated with sulphuric acid showed percentages of infection which compared favorably with those of plants grown from hand-dehulled seeds. With proper facilities for washing, many lots of seeds may be treated with sulphuric acid in a day. Seeds may be washed very easily at the rate of 12 lots an hour. Considerable saving in time and money is gained over the hand-dehulling method.

Data are not numerous enough to justify a positive opinion as to the most satisfactory strength of acid and the proper duration of treatment. Additional experiments have been started in the field

<sup>8</sup> STAKMAN, E. C. DISEASES OF CEREAL AND FORAGE CROPS IN THE UNITED STATES IN 1921. U. S. Dept. Agr., Bur. Plant Indus. Plant Disease Bul., sup. 21, p. 139–254, illus. 1922. [Mimeographed.]

<sup>9</sup> TISDALE, W. H. Op. cit.

<sup>10</sup> FARIS, J. A. Op. cit.

<sup>11</sup> FARIS, J. A. FACTORS INFLUENCING INFECTION OF HORDEUM SATIVUM BY USTILAGO HORDEI. Amer. Jour. Bot. 11: 209. 1924.

at Davis and in the greenhouse at Berkeley, which should yield results indicating the most satisfactory treatment. Because the thickness of the hulls in barley varies with the variety, it may be rather difficult to establish a standard treatment which will be equally satisfactory for all varieties. Preliminary experiments with 10 varieties of barley, to be hereafter reported, seem to indicate that this is not an important matter.

Injury to the seed coat in threshing must be avoided if the seeds are to be treated with acid. The barleys used in these experiments were hand threshed, but preliminary experiments not reported in this paper indicate that seed threshed with a small nursery thresher are practically free from seed-coat injury and may be treated with sulphuric acid without damage from this source.

Covered smut of barley may be controlled by the use of fungicides, but, because this disease is rather sporadic in its occurrence in California, seed treatment is not regularly practiced by many farmers, and consequently considerable losses occur from time to time. Because the acid method of inducing smut infection is comparatively simple and certain, varieties of barley in large numbers may not be tested for resistance to covered smut. Any resistant varieties which are isolated may be used in studying the inheritance of resistance to this disease. Finally, the breeding of resistant varieties suitable for the various barley-growing regions offers a promising means of eventually eliminating covered smut.

#### SUMMARY

Experiments were begun in the greenhouse at Berkeley, Calif., in the fall of 1925, to obtain an easier method of satisfactorily infecting barley with covered smut.

In each experiment barley seeds dehulled by hand before inoculation produced plants with percentages of smut much higher than plants from the natural or hulled seeds.

Seeds subjected to several different treatments with sulphuric acid produced stands equal to those from the hand-dehulled seed, and the resulting plants contained similar percentages of smut.

A very limited field seeding indicates that the acid treatments will give results in the field similar to those obtained in the greenhouse.

Data are not numerous enough at present to indicate the most desirable strength of acid or the proper duration of treatment. Additional experiments in the greenhouse at Berkeley and in the field at Davis have been begun to determine these points.

Experiments to date indicate that it is not necessary to remove the barley hull completely in order to induce satisfactory infection with covered smut. The best stands accompanied by satisfactory infection were obtained when a thin hull remained over the entire kernel or at least over the germ end.

The acid treatment herein described will so simplify the method of inducing covered smut in barley that varieties may be tested in large numbers for resistance to this disease, and the segregation of smut-resistant forms in hybrid generations may be studied.

# THE GROWTH OF BACILLUS RADICICOLA ON ARTIFICIAL MEDIA CONTAINING VARIOUS PLANT EXTRACTS<sup>1</sup>

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## INTRODUCTION

A review of the voluminous literature on legume-nodule bacteria brings out the fact that although the organisms will live and retain their vitality on a large variety of liquid artificial media, they seldom produce thick films and large masses of viscous surface growths except in the presence of certain miscellaneous nitrogen-containing mixtures, such as yeast extract or legume extracts. On a simple synthetic inorganic medium containing, in addition, an available source of energy, growth has usually been reported as small. If nitrates are added to this synthetic medium, the liquid culture usually becomes more cloudy and the gum production may show an increase, but if the sugar used is pure, heavy viscous film growths are seldom if ever obtained. Dawson (1)<sup>3</sup> describes such growths as thick, zoogloealike films. They commonly form from 7 to 10 days after inoculation and may settle to the bottom of the flask a few days later. This paper presents the results of qualitative studies of the effect of plant extracts upon the production of such viscous film growths.

Before proceeding to a discussion of the experimental work, it is well to emphasize that these studies were not undertaken with the idea of developing a more satisfactory medium than those now in common use; neither does the work deal with physiological efficiency, as judged by inoculating power. These subjects have been thoroughly considered by many investigators. Neumann (5) studied about 70 kinds of media, including plant extracts and soil extract. Zipfel (8) observed the growth in various media over a range of temperatures and at different H-ion concentrations. Temple (7) particularly refers to the beneficial effects of adding a small amount of leguminous material to sterile soil for the culture of the organisms. Prucha (6) at the Cornell station has contributed to our information on various phases of culture work. Fred and his associates at Wisconsin have done some very excellent work. One of the pioneers in this line of investigation was Moore (3, 4). Later work from the same laboratory by Kellerman and his associates has added greatly to our knowledge of methods for handling cultures for commercial use. Dozens of other references might be given if space permitted. From all of these studies it is apparent that such media as soil extract, wood ash, and yeast water are excellent for routine work. The writer uses soil-extract media almost exclusively for maintaining stock cultures in an active state and considers them excellent.

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<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 923.

The purpose of this investigation was to determine some of the conditions which favor the production of zoogloelike film growths; the types of plant extracts and other materials which cause them; and finally, if possible, the particular constituent, if any, which is responsible for such unusually vigorous cell multiplication. It was desired to know if the stimulating agent in the extracts is a food or a true stimulant. If a stimulant or a nonnitrogenous food, it was hoped that its separation from plant extracts and its addition to synthetic nitrogen-free media might make conditions for the growth of the organism sufficiently ideal to favor nitrogen fixation on a scale comparable to that obtained with *Azotobacter*. The present paper is of a preliminary nature and deals only with the growth-promoting effects of various plant extracts.

#### MEDIUM USED

In beginning these studies it was necessary to select a suitable combination of inorganic elements, together with an available source of energy, to serve as a basis for all media used throughout the investigations. This mixture should supply as much of all the common elements as is needed for use in heavy growth of the organism, except, of course, those elements which are intended for variables. Plant extracts or other materials, added to this combination, would give more inorganic elements and sugars, but this could not be avoided.

The final selection of the combination of inorganic elements was based both upon the results reported in the literature and upon tests of about 100 combinations made in this laboratory. Space does not permit a detailed description of these experiments but, in brief, it may be stated that *Bacillus radicola* responds particularly to phosphorus, and in the case of some strains, to calcium. A relatively small quantity of the other necessary inorganic elements, other than nitrogen, is sufficient. The combination finally chosen had the following composition, expressed in grams:  $K_2HPO_4$ , 0.2;  $MgSO_4$ , 0.2;  $NaCl$ , 0.2;  $CaSO_4$ , 0.1;  $FeCl_3$ , 0.005;  $CaCO_3$ , 1.0; and  $H_2O$ , 1000.0. This medium, with a suitable source of energy, supplied all of the 10 elements, other than nitrogen, which are considered as most needed by plants; it is wholly synthetic, and furnishes an adequate but not excessive supply of the ingredients.

Several experiments were made to determine the best source of energy for legume bacteria. Of 30 sources tested, 10 were about equally good for a large number of strains of the organism, but some were particularly good for certain strains. Sucrose has been used to the greatest extent by other workers, but at this laboratory it seemed to be no better than several other sugars. It is well to emphasize the fact that the strains differ. The organism used to the greatest extent in these experiments, a red clover culture, showed a slight preference for dextrose, and for this reason this sugar was used in all the plant extracts studied.

No source of nitrogen was included in the media because *Bacillus radicola* is supposed to fix atmospheric nitrogen. In the first experiments reported below no nitrogen was added to the culture medium except that supplied in the plant extracts. Further reference to the part which nitrogen plays in the results will be made in subsequent paragraphs.

## ESTIMATION OF GROWTH

Unfortunately, there is no satisfactory quantitative method for measuring the amount of growth in culture flasks. This statement may seem strange at first, and needs explanation. This study deals with a particular type of growth which can be seen, but which is not easy to measure. The following three possibilities suggested themselves: (1) Obtain the dry weight of the bacterial cells, (2) determine numbers, and (3) separate out the growth and determine the nitrogen.

The determination of dry weight was made in a few instances, but this method was entirely unsatisfactory, especially in the presence of plant extracts. It is very difficult to filter off the growth because of the presence of the bacterial gum, which becomes a portion of the dry weight unless it is removed. But its removal is practically an impossibility, because it is formed as an effluent cell wall of the organism. The presence of  $\text{CaCO}_3$  and miscellaneous plant constituents, some of which may be precipitated by autoclaving or bacterial growth, added to the difficulties.

Bacterial numbers might have been determined, but the writer considers this determination practically worthless for the problem in hand, as well as for most similar problems. Apparently this is the general consensus of opinion among bacteriologists, judging from the discussions at the recent meetings of the First International Congress of Soil Science at Washington. Particularly is it true in the present instance, because the thing to be estimated is a particular type of growth. The heavy viscous films of bacteria on plant-extract media consist largely of masses of giant cells, gonidia, and budding cells held together very closely by the sticky gum. No ordinary system of dilutions would satisfactorily separate the bacteria present in budding clumps, and hundreds or even thousands might form the nucleus of a single colony on a plate. Assuming that the cells could be separated, the method is still unsatisfactory, because the results obtained would depend wholly on the type of the organism predominating on any particular day. The life cycle, as shown by Löhns (2), commonly passes within a few days from motile rods (from 1 to 2 microns in length) to giant cells (from 5 to 30 microns) and then to gonidia (0.3 micron). In the case of plant-extract cultures we may add budding clumps as a common occurrence. By plate counts a gonidium 0.3 micron in diameter would be as important as a gonidangium 30 microns long.

A chemical method of estimating the mass of growth was developed. This consisted in precipitating the bacterial gum, which carries with it the organisms, by the addition of a suitable reagent. Basic lead acetate seemed most effective, although alcohol and various other agents may be used. The precipitate was washed thoroughly and the nitrogen content determined. The method was not satisfactory where plant extracts and other miscellaneous organic materials containing nitrogen were present. In other cases it gave fair results.

In view of the lack of a satisfactory quantitative method, it was decided to judge the growth by the eye only. This gave a better qualitative picture of the abundance of the type of growth under consideration than might be supposed, particularly for the red-clover

strain of the nodule bacteria used. For the purpose of these investigations qualitative results were satisfactory and quickly obtained.

#### METHOD OF STERILIZATION OF PLANT EXTRACTS

In beginning these experiments, the logical plant extract to use in obtaining some of the preliminary data was, of course, the juice from red clover, since the organism used was from the nodules of the roots of that plant. Furthermore, other investigators have repeatedly shown that clover, as well as other legume extracts, greatly stimulates the nodule organism.

Four hundred grams (green weight) of red clover plants, including tops, roots, and nodules, were ground in a meat chopper. To the macerated plants was added 300 c. c. of cold water, and after standing about two hours the mixture was filtered. To one series of 250 c. c. Erlenmeyer flasks containing the inorganic ingredients and dextrose were added in duplicate, aliquot parts of the clover extract corresponding to 3, 6, 12, 30, and 60 gm. of the original clover plant per 100 c. c. of medium. These flasks were plugged with cotton and sterilized in the autoclave at 15 pounds pressure for 20 minutes. The remainder of the original extract was first sterilized by passing it through a sterilized Chamberland-Pasteur filter. After incubation for a week to make certain of sterility, aliquot parts of this extract were then transferred under sterile conditions to flasks containing the sterilized salt-sugar medium already described. Inoculations were made with an active pure culture of the red clover organism and growth was allowed to take place at 26° C. Observations, made at intervals, showed no appreciable difference in growth in corresponding flasks sterilized with and without heat. Growth was good in both cases, and the more clover extract present the heavier the surface film produced, with the exception of the flask receiving the most extract. In this flask early growth was slightly retarded. Growth was exceptionally heavy in this flask, however, by the end of a week. Analyses for total nitrogen by the Kjeldahl method showed no fixation.

#### STUDIES WITH VARIOUS LEGUME EXTRACTS

In order to determine whether the extract made from any given leguminous plant is particularly stimulating for the strain of organisms which normally grows in the nodules of that plant, several additional experiments were made. Included in these tests were some comparisons of the effect of extracts made from the tops, roots, and in some cases from the seeds of a given plant. Incubation was for a period of three weeks at 26° C., and observations were made at frequent intervals. Many of the plant extracts were tested several times under slightly different conditions. The concentration was varied and different batches of the extract were used. It seems scarcely worth while to go into detail regarding all of these separate experiments, but in Table 1 some of the observations are given which particularly show the relation of the concentration of the extract to the results observed. It should be remembered that no nitrogen other than that contained in the extracts was added to any of the flasks.

TABLE 1.—Growth of red clover nodule bacteria on media containing various legume extracts

Source of extract	Grams of green plant material extracted per 100 c. c. of medium	Condition of growth <sup>a</sup>
No plant extract.....	0	x
Red clover tops and roots.....	15	xxxx
Red clover tops.....	2	xx
Do.....	5	xx
Do.....	8	x
Do.....	15	0
Do.....	30	0
Red clover roots.....	2	xxxx
Do.....	5	xxxxx
Do.....	8	xxxxx
Do.....	15	xxxxx
Sweet clover tops.....	2	xx
Do.....	5	xx
Do.....	8	xx
Do.....	15	0
Do.....	30	0
Sweet clover roots.....	2	xx
Do.....	5	xxx
Do.....	8	xxxxx
Do.....	15	xxxxx
Do.....	30	xxx
White clover tops.....	4	0
Do.....	12	0
Do.....	16	0
Navy bean seeds.....	.4	xx
Do.....	.7	xxx
Do.....	1.8	xxv
Do.....	3.6	xxxx
Do.....	7.2	xxxx
Do.....	10	xxxx

<sup>a</sup> 0=no growth; x=slight growth; xx=fair growth; xxx=medium growth; xxxx=good growth; xxxxx=very good growth.

The data in Table 1 indicate very definitely that the red clover organism does not show a greater response to red clover extract than to certain other legume extracts, such as those from sweet clover roots or from navy bean seed. Another point of considerable significance is the marked difference between the extracts of the tops and the roots of leguminous plants. The extracts of both red clover and sweet clover tops produced slight stimulations at the lower concentrations, but prevented all growth at the 15 gm. and 30 gm. rates. Even at the lowest concentration of 4 gm., the extract of white clover tops prevented growth. The appearance of the cultures suggested that poor growth was probably due to the presence of a toxic material. Several chemical tests were made in order to determine definitely if such were the case, and it was found that an extract from red clover tops, after precipitation with alcohol and removal of the precipitate, was very active in increasing bacterial growth. No attempt was made to identify the toxic material. Analyses of a portion of the cultures listed in Table 1 showed no gains in nitrogen.

#### STUDIES WITH VARIOUS NONLEGUME EXTRACTS

In order to determine if juices from nonlegume plants also favor luxuriant growth of *Bacillus radiculicola*, several vegetables and other plants were used and tested in the same way without the addition of any nitrogenous compounds other than those in the plant juice. Extractions were made by grinding the materials, adding a little

CaCO<sub>3</sub>, boiling the mixture for a few minutes, and then filtering it. The results are shown in Table 2.

TABLE 2.—Growth of red clover nodule bacteria on media containing various non-legume extracts

Source of extract	Grams of green plant material extracted per 100 c. c. of medium	Condition of growth <sup>a</sup>
No plant extract.....	0	x
Banana.....	2	x
Do.....	5	x
Do.....	8	xxx
Do.....	15	0
Do.....	30	0
Beet roots.....	2	xx
Do.....	5	xx
Do.....	8	xx
Do.....	15	xx
Do.....	30	xx
Do.....	50	xx
Blue grass tops.....	6	xxx
Do.....	12	xxxx
Do.....	24	xxxxxx
Cabbage.....	2	xy
Do.....	5	xxx
Do.....	8	xxx
Do.....	15	xxxxx
Do.....	30	0
Carrot.....	11	xxax
Do.....	22	xxxxx
Green corn tops.....	10	xxx
Do.....	20	xxxx
Do.....	60	xxxxx
Lettuce.....	2	xy
Do.....	5	xy
Do.....	8	xxx
Do.....	15	xxxx
Do.....	30	xxxxx
Orchard grass tops.....	2	xx
Do.....	5	0
Do.....	8	0
Do.....	15	0
Do.....	30	0
Orchard grass roots.....	2	x
Do.....	5	xy
Do.....	8	xxx
Do.....	15	xxxx
Parsnip.....	10	xxx
Do.....	20	xxx
Potato.....	9	xx
Do.....	18	xxx
Do.....	45	xxxx
Spinach.....	8	x
Do.....	1.5	x
Do.....	4	xx
Do.....	8	xxxx
Do.....	12	xxxxx
Do.....	16	xy
Sweet potato.....	3	xx
Do.....	6	xx
Do.....	15	x
Do.....	21	0
Do.....	42	0
Turnip.....	11	x
Do.....	22	xx

<sup>a</sup> 0=No growth; x=slight growth; xx=fair growth; xxx=medium growth; xxxx=good growth; xxxxx=very good growth.

It will be observed that the extracts from a large number of plants favor the growth of legume bacteria, there being little difference between many nonlegumes and legumes. When used in the optimum concentrations, extracts of orchard-grass roots, lettuce, cabbage, carrot roots, spinach, corn tops, and bluegrass produced growths

comparable to those produced by legume-root extracts. Banana, potatoes, and parsnip extracts also produced good growths, but less than the above-named plants. Results obtained from extracts from orchard grass tops, sweet potato, and turnip were the poorest. Here the same observations may be made as are indicated in Table 1, namely, that some extracts prevent growth if used in too high a concentration, probably because of the presence of toxic compounds. Here again analyses failed to show any nitrogen fixation by *Bacillus radicola*.

When this work was undertaken the writer expected to find that extracts of leguminous plants would produce much better media than those of nonlegumes, if reinforced with sugar and the necessary inorganic salts; but this did not prove to be true. If the activity were limited to plants which produce nodules, then it would be logical to infer that the stimulant or food plays an important rôle in symbiosis. The experiments reported here probably have little bearing on symbiosis.

#### IMPORTANCE OF NITROGEN

Since the above experiments had failed to demonstrate any powers of nitrogen fixation by the red-clover organism under the conditions of the experiment, it seemed very desirable to compare the growths resulting from several plant extracts with an available source of nitrogen added. Another series of experiments was, therefore, carried out, giving a direct comparison between the growths in the presence and in the absence of a nitrogenous salt. One hundred milligrams of  $\text{KNO}_3$  was used per 100 c. c. of medium, because several tests, which can not be included here, had shown this to be a very satisfactory source of nitrogen. Because of the large number of extracts tested simultaneously, only two concentrations could be used; but with the previous work to serve as a guide, it was not difficult to select concentrations that would bring out the salient points.

All extracts were prepared by grinding 100 gm. of the plant material, adding about 5 gm. of  $\text{CaCO}_3$  and 200 c. c. of water, and autoclaving at 15 pounds pressure for 20 minutes. After filtration the extracts were reduced to dryness on a steam bath, again diluted, filtered, and made up to 500 c. c. Extreme difficulty was experienced in the filtration of some of the extracts, but by using both the centrifuge and suction filter, where necessary, a reasonably clear extract was obtained in all cases. Two extracts were made in the case of yeast, one of a good commercial yeast in cake form and the other of yeast cells grown on a synthetic medium. Table 3 gives the comparative growths, as determined by observation, for each of the extracts under the various conditions of the tests. In considering these results it should be remembered that the figures in the table showing the quantity of extract used are based on the green weights of the plants. If the tests had been on the dry basis the relative activity would have been quite different. These experiments were not planned, however, as quantitative tests, but merely to gain a general idea of the stimulating properties of various extracts used in the presence and in the absence of an available nitrogenous salt.

TABLE 3.—Comparative tests of various plant extracts without and with added nitrogen

Source of extract	Grams of green plant material extracted per 100 c. c. of medium	Condition of growth without $\text{KNO}_3^1$	Condition of growth with $\text{KNO}_3^2$
No plant extract.....	0	x	x
Alfalfa seeds.....	2	xxx	xxxx
Do.....	10	xxx	xxxxx
Apple.....	2	x	x
Do.....	10	x	x
Banana.....	2	x	x
Do.....	10	xxx	xxxx
Beet roots.....	2	x	x
Do.....	10	x	xx
Cabbage.....	2	xx	xx
Do.....	10	xxx	xxxx
Carrot.....	2	x	x
Do.....	10	xxx	xxxxx
Carrot tops.....	2	x	x
Do.....	10	0	0
Clover seeds.....	2	xxx	xxxx
Do.....	10	xxx	xxxxx
Corn meal.....	2	x	xx
Do.....	10	xx	xxx
Cowpea seeds.....	2	xxx	xxx
Do.....	10	xxxx	xxxxx
Lettuce.....	2	x	x
Do.....	10	xxx	xxxxx
Orange.....	2	xx	xx
Do.....	10	xxx	xxxx
Parsnip.....	2	xx	xx
Do.....	10	xxx	xxxx
Potato.....	2	xx	xx
Do.....	10	xxx	xxxx
Soy-bean seeds.....	2	xx	xxx
Do.....	10	xxxx	xxxxx
Spinach.....	2	xx	xx
Do.....	10	xxx	xxxxx
Sweet potato.....	2	x	x
Do.....	10	xx	xx
Tomato.....	2	xx	xx
Do.....	10	xxx	xxxx
Turnips.....	2	x	x
Do.....	10	xx	xx
Yeast, cultivated.....	2	x	xx
Do.....	10	x	xx
Yeast, commercial.....	2	xxx	xxxx
Do.....	10	xxx	xxx

\* 0=no growth; x=slight growth; xx=fair growth; xxx=medium growth; xxxx=good growth; xxxxx=very good growth.

From the data in Table 3 it will be observed that the addition of  $\text{KNO}_3$  to culture flasks which contained the various plant extracts usually increased the growth and was in no case injurious, regardless of the quantity of nitrogen added. Even the growths in the presence of the extracts of some plant products, like clover seed and alfalfa seed, which contain an abundance of nitrogen, were greater with nitrogen added. This shows the necessity for the addition of a source of nitrogen in all cases where tests such as those reported here are to be made. Table 3 also shows that plants which represent very widely different species contain excellent sources of food for legume bacteria. Orange juice, tomatoes, lettuce, and spinach would show an especially high activity if comparisons were made on a dry basis. The least stimulating extracts were those from beets, sweet potatoes, turnips, apples, and carrot tops. No experiments were made to determine if the low activity of these extracts was due to the presence of toxins, as was

previously found to be true in the case of red clover tops. In these experiments yeast isolated in pure culture from a commercial yeast cake and grown on a synthetic medium failed to show any appreciable synthesis of the bacterial stimulant or food; on the other hand, an extract of the commercial yeast cake was very active. The activity can probably be attributed to the original ingredients used in preparing the commercial product.

#### SUMMARY

A study of the growth-promoting properties of plant extracts for legume-nodule bacteria is reported. The results given were obtained with a red clover organism, this culture having shown a greater response in preliminary tests than any other strain used. It was found that certain strains of the organism, which ordinarily make a poor growth on a simple sugar-salt medium, produce luxuriant growth in the presence of the juices of certain plants. Extracts were prepared from 26 different plant materials, both legumes and non-legumes, including vegetables, fruits, and field crops. The extracts from practically all of these increased bacterial growth to some extent, at least when used in the proper concentration; but the activity varied widely for different plants and even for different parts of the same plant. Extracts of legume roots particularly favor the growth of *Bacillus radicola*. Of the nonlegume extracts, the best were from lettuce, cabbage, carrot, spinach, tomato, green corn tops, bluegrass, and orange. The bacterial food value of any extract of any given species of plant varies widely with different samples, depending largely on the stage of maturity.

These studies have shown very definitely that there is a wide difference in the response of various strains of legume bacteria to plant-extract media. Of 30 strains taken from as many different species of plants, the clover-alfalfa group of organisms gave the largest increases in growth. Many strains will, however, show increasing response to plant extracts after several transfers on the same medium.

A report of experiments to determine the nature of the plant constituents which are primarily responsible for the production of such abundant growths of certain strains of legume-nodule bacteria, when the extracts are added to artificial liquid media, will be given in a subsequent paper.

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# RELATION OF TEMPERATURE TO GROWTH OF *PENICILLIUM ITALICUM* AND *P. DIGITATUM* AND TO CITRUS FRUIT DECAY PRODUCED BY THESE FUNGI<sup>1</sup>

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## INTRODUCTION

Two of the most important decay-producing fungi in citrus fruits are *Penicillium italicum*, which produces blue contact mold, and *P. digitatum*, which produces common green mold. The investigation of these fungi in relation to temperature had for its purpose the gaining of information not only on the rate of growth from day to day on artificial media but also on the rate and percentage of decay produced in orange fruits. The results were found to show interesting differences in rate of decay between the stem end and the stylar end of the fruit.

## METHODS

The cultures of the two fungi were grown on glucose-potato agar in Petri dishes 90 cm. in diameter, each dish containing 10 c. c. of the medium. Minute drops of water containing a few spores were placed on this medium at the center of each dish, and the dishes were promptly placed in the temperature chambers. The fluctuations in each chamber were about one-half degree centigrade. The diameter of the circular mycelial mat in each dish was measured at intervals of two days.

The fruits used in the experiments were Valencia oranges from Orange County, Calif., picked during the latter part of August, 1925. They had been washed in the packing house and were prepared by being rinsed with 95 per cent alcohol, injured with uniform clipper cuts approximately one-half centimeter in diameter at both the stem and stylar ends, and inoculated by brushing spores on the injuries. The clippers were dipped in alcohol and flamed before each cut. The spores were taken from decayed fruit that had been inoculated with pure cultures of *Penicillium italicum* and *P. digitatum* and that showed no outward signs of contamination with other fungi. The fruit was then soaked four minutes in water at 95° and 115° F., allowed to drain and dry on wire racks, and wrapped in individual orange wrappers. Lots of 10 fruits each were placed in net bags for storage at different temperatures.

Separate uninoculated lots of eight fruits each were held at each temperature and weighed at intervals to determine the shrinkage and to serve as checks against the relative humidity readings. Temperatures of other individual fruits were taken by inserting mercury thermometers. A hair hygrometer was used in taking the relative humidity record.

<sup>1</sup> Received for publication July 27, 1927; issued December, 1927. Paper No. 167, University of California, Graduate School of Tropical Agriculture and Citrus Experiment Station.

Except in cases in which decay had started at both ends or the decayed area was so large as to make further keeping impracticable, the fruit was returned to the temperature chambers for further observation. After 12 days at the different temperatures, the lots of fruit were removed from the several chambers and placed in moist jars at about 70° F. for further study of the development of decay.

Table 1 gives the air and fruit temperatures in the various maintained-temperature compartments for the first six days.

TABLE 1.—Fruit temperatures of California Valencia oranges stored at different air temperatures

Day of storage	Fruit temperature (° F.) at average ° air temperature of—						
	50.0° F.	57.7° F.	66.8° F.	74.8° F.	80.6° F.	86.0° F.	88.7° F.
First.....	80.7	80.7	80.7	80.7	80.7	80.7	80.7
Second.....	56.5	63.3	70.2	73.9	78.8	85.0	89.1
Third.....	56.0	63.0	70.2	76.0	80.0	85.5	89.7
Fourth.....	54.0	66.0	69.2	-----	-----	86.0	91.8
Fifth.....	55.0	64.0	70.0	76.0	81.0	86.0	90.0
Sixth.....	54.0	64.0	69.0	76.0	81.0	85.0	88.5

° Average for 12 days.

Table 2 gives average air temperatures, relative humidity, and weight lost by the fruit during the first eight days.

TABLE 2.—Relative humidity and loss in weight of California Valencia oranges stored at different air temperatures

Item	Average air temperature °						
	50.0° F.	57.7° F.	66.8° F.	74.8° F.	80.6° F.	86.0° F.	88.7° F.
Relative humidity °.....	41.6	53.3	65.4	77.7	85.1	89.7	83.7
Loss in weight per 100 gm. fruit, ° gm.....	1.22	1.39	1.55	-----	1.6	1.95	4.85

° Average for 12 days.

° Average for 8 days.

GROWTH RATE OF PENICILLIUM ITALICUM AS INFLUENCED BY TEMPERATURE

TABLE 3.—Growth of *Penicillium italicum* in orange fruits and in glucose-potato agar at different temperatures

AVERAGE DIAMETER OF DECAYED SPOTS ON ORANGES								
Fruit end and culture	Days	50° F.	57.5° F.	66.8° F.	74.8° F.	80.6° F.	86° F.	88.7° F.
		Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
Stem end.....	4	1.2	12.5	20.4	28.9	23.7	16.2	10.1
	6	-----	28.7	43.1	43.7	41.0	21.1	12.2
	8	24.2	41.4	57.7	59.5	57.8	24.5	12.3
	12	48.0	78.0	-----	-----	-----	36.1	-----
Stylar end.....	4	0.0	1.2	8.5	11.5	8.2	6.5	0.0
	6	-----	18.5	26.0	27.0	16.8	9.7	0.0
	8	9.8	26.3	39.7	39.8	24.3	16.8	0.0
	12	27.8	58.2	-----	-----	-----	24.5	-----

DIAMETER OF FUNGUS MYCELIAL MAT ON GLUCOSE-POTATO AGAR								
Culture.....	4	11.0	21	24	28	26	12	6
	6	18.5	31	35	37	32	14	7
	8	26.0	40	44	49	36	18	9
	12	35.0	57	67	65	49	20	11

The data in Table 3 and Figure 1 show that *Penicillium italicum* when grown on glucose-potato agar made somewhat the same general response to temperature as when invading the orange fruits. The curves in Figure 1 all have the same optimum point. There is one interesting difference, however, in that the growth on the artificial medium was proportionally faster than on the fruit at the low temperatures, especially in the fruit held four days. From about 75° F. up, the rate on the artificial medium was about the same as at the stem-end half of the fruit. In eight days the decayed areas at the stem-end were almost as large as the mycelial mats in cultures at the lower temperatures and exceeded their growth from about 57° F. up. The average rate of enlargement of decayed spots near the stylar end was very much slower than with those near the stem end at all temperatures during the first eight days. In 12 days the enlargement of decayed spots at the stylar end closely approached that of mycelial mats in cultures. As is seen from Table 1, the fruits at the lower temperatures were slow in cooling down to the temperature of the air in the chamber. This difference in rate of cooling would not seem to account for the lag in enlargement of rot areas as compared with the advance in artificial cultures. In fact a greater lag would be expected if the fruit had cooled as rapidly as the cultures. This difference is probably due to a resistance in the living cells of the fruit to the advance of the invading organism, which resistance is relatively greater at low than at higher temperatures. These data indicate that temperatures between about 65° and 80° are conducive to a rapid rate of decay from *P. italicum* and that at temperatures below and above this range the rate is rapidly retarded. A temperature of 90.5° F. appears to be near the upper limit for growth. A temperature of 50° F. appears to be considerably above the lower limit, but greatly retards the rate of development of decay.

A marked difference in rate of decay is shown between the stem and the stylar half of the fruit. The shape of the graphs of the enlargements, however, is very similar, indicating the same optimum point near 75° F. with a retardation of rate at lower and higher temperatures in much the same way in both cases.

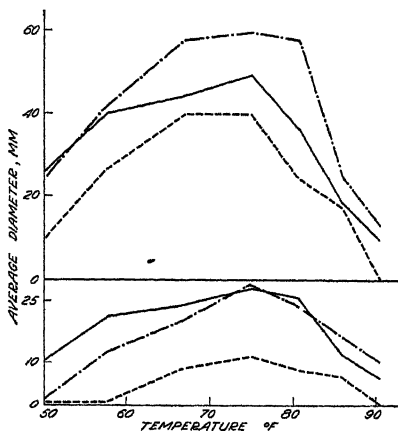


FIG. 1.—Growth of *Penicillium italicum* in relation to temperature and to location of inoculation. (Lower graphs, 4-day growth; upper graphs, 8-day growth)

At stylar half of fruit — · · · · ·  
At stem-end half of fruit — — — — —  
On glucose-potato agar —————

# GROWTH RATE OF *PENICILLIUM DIGITATUM* AS INFLUENCED BY TEMPERATURE

TABLE 4.—*Growth of Penicillium digitatum in glucose-potato agar at different temperatures*

Days	Diameter of mycelial disks at—						
	50° F.	57.5° F.	66.8° F.	74.8° F.	80.6° F.	86° F.	90.5° F.
	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.	Mm.
Second.....	0	6.7	13.0	14.5	15	5.0	0
Fourth.....	6	20.0	35.0	38.0	30	5.0	0
Sixth.....	13	33.0	53.5	56.0	37	6.5	0
Eighth.....	22	53.0	75.0	76.0	51	7.5	0

The data in Table 4 and Figure 2 indicate that *Penicillium digitatum* also has an optimum temperature somewhere near 75° F., and possibly, as judged from the shape of the graphs, the optimum is a little lower than that of *P. italicum*. The retardation of growth in passing from the optimum to lower and higher temperatures appears to be relatively more rapid than with *P. italicum*. The upper limit appears to be below 90.5° F., since no growth took place at that temperature. At 50° F. a marked retardation is evident. The rate of spread of this fungus was so rapid that measurements on the fruit corresponding to those for *P. italicum* were not feasible.

## DECAY OF FRUIT BY *PENICILLIUM ITALICUM*

TABLE 5.—*Penicillium italicum* decay on inoculated California Valencia oranges stored at different temperatures, showing relative susceptibility of stem and stylar end of fruit

Temperature of air (°F)	Number of fruits	Percentage of injuries showing decay after—							
		4 days		8 days		12 days		28 days	
		Stem end	Stylar end	Stem end	Stylar end	Stem end	Stylar end	Stem end	Stylar end
50.....	10	30	0	100	60	100	90	-----	-----
	10	0	0	70	20	100	100	-----	-----
	10	0	0	80	10	100	90	-----	-----
Average.....		10	0	83.3	30	100	93.3	-----	-----
57.5.....	10	60	10	100	90	100	100	-----	-----
	10	90	60	100	100	-----	-----	-----	-----
	10	60	20	100	100	-----	-----	-----	-----
Average.....		80	30	100	96.7	-----	-----	-----	-----
66.8.....	10	100	60	100	100	-----	-----	-----	-----
	10	100	70	100	100	-----	-----	-----	-----
	10	60	30	90	90	-----	-----	-----	-----
74.8.....	10	100	60	100	100	-----	-----	-----	-----
80.6.....	10	100	60	100	70	-----	-----	-----	-----
Average.....		92	56	98	92	-----	-----	-----	-----
86.....	10	100	50	100	60	100	70	-----	-----
	10	90	0	100	0	100	0	-----	-----
	10	90	30	100	30	100	30	-----	-----
Average.....		93.3	26.7	100	30	100	33.3	-----	-----
88.7.....	10	0	0	0	0	0	0	40	50
	10	40	10	50	50	50	50	60	70
	10	60	10	90	40	90	40	90	40
Average.....		33.3	6.7	46.7	30	46.7	30	63.3	53.3

Differences in temperature produced noticeable differences in the quantity and location of decay and in the time required for the injuries to soften. Table 5 gives the percentage of injuries showing decay on Valencia orange fruits inoculated with *Penicillium italicum* at the stem and stylar end. Figure 3 contains a graphical presentation of the average percentages. Practically all the fruit held at temperatures ranging from 57.5° to 86° F. decayed in 4 or 8 days, but when held at 50° total decay required from 8 to 12 days. At 88.7° the amount of decay was below 50 per cent at the end of 12 days, and increased to only a little over 60 per cent during 16 additional days in 70° moist jars. This fact suggests a possible killing of some spores by the high temperature. In fact, in one experiment 90 per cent of the fruit started to soften at the injuries during the first four days, but all of these spots became dry and showed no advance or spore formation during the rest of the high-temperature storage period, and only about 50 per cent of the inoculations developed decay after the fruit was removed to 70° moist jars.

The difference in amount of decay present in injuries at the stem and stylar ends is found mainly in the period of maximum production of decay. At temperatures from 57.5° to 80.6° F. practically all fruits were decayed at both ends in 8 days, although the maximum production of decay at the stem end occurred during the first 4 days while that at the stylar end occurred during the second 4 days. At 50° practically all the fruits were decayed at both ends in 12 days, although the maximum production of decay occurred at the stem end during the second 4-day period while that at the stylar end occurred during the third period. The fruit held at 86° showed decidedly less decay at the stylar end than at the stem end and the difference was consistent throughout three experiments. At 88.7° the percentage of decay was slightly less at the stylar end than at the stem end, and the maximum development of decay at the stylar end came during the 4-day period following that at which it occurred at the stem end.

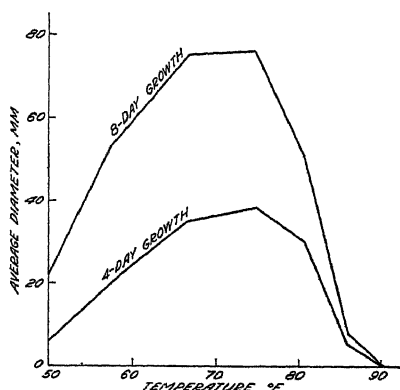


FIG. 2.—Growth of *Penicillium digitatum* on glucose-potato agar in relation to temperature

#### DECAY OF FRUIT BY *PENICILLIUM DIGITATUM*

Table 6 gives the percentage of injuries showing decay due to *Penicillium digitatum* at stem and stylar end of Valencia oranges. These averages are graphically presented in Figure 3. The decay of orange fruits by *P. digitatum* apparently is affected more by differences in temperature than is that by *P. italicum*. There appears to be relatively less decay by *P. digitatum* at temperatures above 80.6° F. and below 66.8°. This difference in general parallels the relative difference in rate of growth of the two organisms at the various temperatures. At temperatures of 66.8° to 80.6° practically



At temperatures ranging from 66.8° to 86° F. decay at the stylar end was more rapid with *Penicillium digitatum* than with *P. italicum*. As much decay was produced at stylar-end injuries by *P. digitatum* in the same time at temperatures of 66.8° to 86° as was produced at stem-end injuries. At temperatures of 50° to 57.5° the maximum production of decay at the stylar-end injuries occurred one 4-day period later than maximum decay at the stem-end injuries.

#### SUMMARY

*Penicillium italicum* and *P. digitatum*, the two fungi most commonly producing soft rot in citrus fruits, are in general affected similarly by different temperatures.

On culture media as well as on orange fruits both appear to have nearly the same optimum temperature for development. The differences, however, between the rate of growth at the optimum as compared with the rates at higher and lower temperatures are greater with *Penicillium digitatum* than with *P. italicum*.

In most cases the rate of development of decay was much more rapid at the stem end than at the stylar end of the orange fruits. The percentage of decay in most cases was also greater from injuries near the stem end than from equal injuries near the stylar end.

In lots of fruit inoculated with *Penicillium italicum* and held, respectively, at 66.8°, 74.8°, and 80.6° F., nearly all fruits showed decay in 4 days, while lots held at 86° and 57.5° showed similar decay in 8 days, and lots held at 50° showed similar decay in 12 days.

In lots inoculated with *Penicillium digitatum* much the same general relation of temperature to time and percentage of decay was noted except that apparently the temperatures above and below the optimum had a greater relative inhibiting effect than in the lots inoculated with *P. italicum*.



# THE RESISTANCE OF CERTAIN VARIETIES OF WINTER WHEAT TO ARTIFICIALLY PRODUCED LOW TEMPERATURES<sup>1</sup>

By DONALD D. HILL, *Instructor in Agronomy*, and S. C. SALMON, *Professor of Farm Crops, Kansas State Agricultural College*

## INTRODUCTION

As is well known, injury to wheat by winterkilling results in severe losses in the winter-wheat-producing regions of the United States. The exact loss is difficult to estimate because it is so easily confused with injury from other causes such as drought, soil blowing, Hessian fly, etc. According to estimates made by the United States Department of Agriculture,<sup>2</sup> the average loss from frosts or freezing for the 14-year period 1909 to 1922 was 3.5 per cent. According to the same authority, the average acreage abandoned for the 23-year period from 1901 to 1923 was approximately 10 per cent. Much, but not all, of this abandonment was due to winterkilling.

The problem of winter hardiness is somewhat more important than these estimates suggest, since there is involved not only the actual loss but also the potential gain that might be derived from growing winter wheat in what is now regarded as spring-wheat territory were it possible to secure varieties that would survive the winters.

Varieties are known which are considerably more hardy than those generally used in commercial production. They have certain defects, however, such as late maturity, inferior quality, and low yields, which more than offset their greater hardiness for most conditions.

These facts suggest that breeding wheat for cold resistance may properly be considered a project of major importance and worthy of serious attention. In such a project the determination of the relative hardiness of varieties now in existence, as well as of any new ones that may be produced, is of first importance. This, however, is a difficult matter because of the variations in seasons as well as other sources of error. Some winters are so mild that the least hardy varieties survive and others are so severe that all are killed. In either case, nothing is learned as to their relative winter hardiness. At the Kansas station, for example, nine successive winters have been so mild that no information on relative winter hardiness has been obtained. It is known from previous experience, though, that a variety to be generally satisfactory in Kansas must possess a reasonably high degree of winter hardiness. The importance of the problem is further emphasized by the fact that at the present time approximately 4,000,000 acres of Blackhull, a variety of questionable winter hardiness, is being grown in this State. It is believed that the success of this variety has been partially due to the mild winters prevailing in Kansas during the past few years. These facts suggest the urgent need of some method of determining the relative winter hardiness more quickly and more certainly than can be done by field trials.

<sup>1</sup> Received for publication Oct. 11, 1927; issued December, 1927. Contribution No. 172, Department of Agronomy, Kansas State Agricultural College.

<sup>2</sup> UNITED STATES DEPARTMENT OF AGRICULTURE. AGRICULTURAL STATISTICS. BREAD GRAINS. U. S. Dept. Agr. Yearbook 1923: 601-661. 1924.

Many attempts have been made to correlate cold resistance with some easily observed morphological or physiological character. The relation of winter hardiness to the size of the cells, the habit of growth, osmotic pressure of the cell sap, the water and sugar content of the tissue, the hydrophilic colloids of the protoplasm, and other characters have been studied. No results of great value have been secured, so far as finding a practical means of detecting winter-hardy varieties or strains is concerned.

### EXPERIMENTAL DATA

During the past year an attempt was made at the Kansas Agricultural Experiment Station to determine the feasibility of artificially freezing plants as a means of determining their relative hardiness. The general plan was to grow varieties of known hardiness and subject them to low temperatures under controlled conditions. The low temperatures were secured with a carbon dioxide direct-expansion refrigeration plant, in which temperatures were controlled automatically. In a preliminary trial 25 varieties were studied, but it seemed desirable in later experiments to limit the study to 10, so selected as to secure a rather wide range in winter hardiness. The plants were grown in the greenhouse and subjected to various degrees of cold for different periods of time with and without previous hardening and with various degrees of soil moisture. Part of the plants were grown in greenhouse flats and part in 4-inch clay pots. After freezing, the plants were thawed gradually and then placed in the greenhouse with suitable temperatures for growth. The apparent injury to the plants was recorded from 5 to 7 days after freezing and the final survival 10 to 14 days later. About 100 trials of 100 plants each were made, of which about 75 per cent were hardened before freezing. Table 1 gives the average survival of about 50 trials in which the plants were hardened for various periods of time previous to freezing. The relative average survival of nine of these varieties grown in the uniform winter-hardiness nurseries of the United States Department of Agriculture for the two years 1925-26 and 1926-27 is also given for comparison.<sup>3</sup>

TABLE 1.—Average survival of wheat varieties expressed as a percentage of Kharkof

Variety	Artificial refrigeration		Uniform winter-hardiness nursery: Average 1925-26 and 1926-27	
	Survival	Rank	Survival	Rank
	<i>Per cent</i>		<i>Per cent</i>	
Minhardi.....	96	4	123.9	1
Buffum.....	104	1	122.5	2
Minturki.....	88	5	116.5	3
Kharkof.....	100	2	100.0	4
Kanred.....	100	2	95.9	5
Harvest Queen.....	80	6	87.7	6
Tenmarq.....	73	7	85.6	7
Blackhull.....	57	8	77.2	8
Fulcaster.....	54	9	71.5	9
Nebraska No. 28.....	47	10		

<sup>3</sup> Data kindly furnished by J. Allen Clark, J. H. Martin, and J. H. Parker.

Nebraska No. 28 was not included in the uniform winter-hardiness nurseries in these two seasons, but from previous work <sup>4</sup> and from data secured at Manhattan it is known that this variety is relatively nonwinter hardy as measured by field tests. It may be seen that excepting the two very hardy varieties, Minhardi and Minturki, the results for the two methods of testing are in very close agreement. In general the failure to agree in all respects is not surprising, considering (1) the variation in the comparative hardiness from year to year and from place to place in the field trials; (2) the fact that in field trials, factors other than degree of cold undoubtedly played a part; and (3) that in the artificial refrigeration trials, the plants were only partially hardened. The periods of hardening were in no case longer than 19 days, and often only 3 or 4 days with temperatures seldom or never below 20° F.

The usefulness of artificial refrigeration was also shown in trials comparing Kanred and Blackhull. The popularity and large acreage of the latter variety in the southern Great Plains suggested extensive trials with it in comparison with Kanred. Plants of these two varieties were dug up from field plots in January, placed in small greenhouse flats, one-half of each flat being Kanred and the other Blackhull, and subjected to a minimum temperature of approximately -10° F. for various periods of time. Table 2 gives the detailed results.

TABLE 2.—*Relative survival of Kanred and Blackhull wheat when subjected to low temperatures*

Lot No.	Variety	Hours frozen	Apparent injury at end of—		Survival
			7 days	15 days	
			Per cent	Per cent	Per cent
1.....	{ Kanred.....	6	10	0	100
	{ Blackhull.....	6	10	0	100
2.....	{ Kanred.....	12	20	30	100
	{ Blackhull.....	12	50	80	40
3.....	{ Kanred.....	15	15	20	100
	{ Blackhull.....	15	50	98	2
4.....	{ Kanred.....	18	30	30	100
	{ Blackhull.....	18	50	100	0
5.....	{ Kanred.....	21	50	70	60
	{ Blackhull.....	21	80	100	0
6.....	{ Kanred.....	24	60	90	10
	{ Blackhull.....	24	85	100	0

It will be observed that the results are in close agreement with what is known of the relative survival of these varieties as determined in the uniform winter-hardiness nurseries. Altogether the results of the artificial refrigeration trials indicate that the method is a very promising one for studying the relative hardiness of different varieties.

A very interesting and equally important factor in the study of winter hardiness is the degree of hardening to which the plants are subjected before they are frozen. This is illustrated in Table 3, in which are given the results of 17 trials with greenhouse-grown plants subjected to freezing temperatures without previous hardening.

<sup>4</sup> CLARK, J. A., MARTIN, J. H., and PARKER, J. H. COMPARATIVE HARDINESS OF WINTER-WHEAT VARIETIES. U. S. Dept. Agr. Circ. 378, 20 p., illus. 1926.

TABLE 3.—Average survival in 17 freezing trials with unhardened plants, expressed as a percentage of Kharkof

Variety	Percentage survival	Rank
Minhardi.....	66	6
Buffum.....	74	3
Minturki.....	69	5
Kanred.....	100	1
Kharkof.....	100	1
Harvest Queen.....	71	4
Tenmarq.....	46	9
Blackhull.....	54	7
Fulcaster.....	47	8
Nebraska No. 28.....	28	10

The average survival of the three varieties Minhardi, Buffum, and Minturki, known to be hardiest under field conditions, was but little better than that of such varieties as Blackhull, Harvest Queen, and Fulcaster, known to be relatively low in the winter-hardiness scale. This suggests not only that hardening is very important in determining survival but also that different varieties react very differently in this respect. It is probable that had the experimental plants been more thoroughly hardened before freezing, the results presented in Table 1 would have been much more favorable for those known to be winter-hardy under field conditions. It seems quite likely that hardiness is due to the ability of certain varieties to build up, in some way, a high degree of protection from cold, whereas other varieties lack this ability. Observations made on individual lots support this idea in that hardy varieties appeared to the best advantage in those cases where hardening was more nearly complete.

The effect of differences in hardening is also indicated by data obtained from freezing individual lots of Kanred which had either not been hardened or had been subjected to two conditions of hardening, namely, mild or greenhouse hardening and field hardening. The data presented in Table 4 show how little of the maximum hardiness had been built up by the greenhouse hardening. The difference between the mild-hardened and the field-hardened plants is especially marked. In view of the behavior of the hardier varieties in these trials it is not unreasonable to suppose that the difference might be still greater with other varieties.

TABLE 4.—Freezing trials with Kanred unhardened and hardened for different periods

Treatment	Mean temperature	Period of freezing	Survival
	° F.	Hours	Per cent
Unhardened.....	0	2.5	70
Mild hardened.....	0	5.0	70
Field hardened.....	-10	18.0	100
Do.....	-10	21.0	60

In a general way, it appears that the 10 varieties may be placed, tentatively at least, in three groups. (1) Varieties which are relatively hardy when thoroughly hardened before freezing. This group

includes Minhardi, Minturki, and Buffum. (2) Varieties which are relatively hardy when hardened before freezing but which also carry a relatively high degree of hardiness in the unhardened condition. In this class are Kanred, Kharkof, and possibly Harvest Queen. (3) Varieties which are relatively nonhardy regardless of whether they are hardened prior to freezing. Such varieties are Blackhull, Nebraska No. 28, Fulcaster, and probably Tenmarq.

The relation of the moisture content of the soil to resistance to freezing injury was also investigated in a number of trials. In all cases, it was found that plants growing in a dry soil were injured much more severely than similar plants in a wet soil. Plants dried to the point of wilting and watered just before freezing survived as well as those which had been abundantly supplied with water at all times. This is easily explained by the high specific heat of water which in a wet soil prevents a rapid change in temperature, the net result being that those plants in dry soil are exposed to a lower temperature than those in wet soil. These results confirm the general observation that winterkilling is frequently more severe in dry soils than in those well supplied with moisture, excepting, of course, those cases where winterkilling is a result of heaving or smothering rather than of low temperature. These relations would seem to be particularly important in determining artificially the relative winter hardiness of varieties. It is rather difficult to maintain a uniform moisture content under greenhouse conditions, and if this matter is not carefully considered, differential results in freezing may be due as much to differences in moisture content of the soil as to differences in varieties



# RELATION BETWEEN WATER AND POTASH IN PLANT PRODUCTION <sup>1</sup>

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## INTRODUCTION

The beneficial effects of potash fertilizers in dry seasons are mentioned by Hall in his description of the Rothamsted experiments.<sup>3</sup> The relation between potash fertilizers and the economical use of water by crops has also been discussed by Von Seelhorst.<sup>4</sup> Hall considers that plentiful potash prolongs the growth of the plant and offsets the ripening action of the phosphoric acid, which in the absence of potash acts prematurely and the action is intensified by the heat and dryness. The opinion of Von Seelhorst is that the potash effect is in accord with the law of the minimum, and he shows that if nitrogen or phosphorus is the scarce element, its addition will likewise produce a more efficient use of water by the plant.

As part of a study of the availability of the potash naturally present in some typical soils, observations were made on the relations between the water supply and potash supply by means of pot experiments with soy beans and Japanese millet. These two crops are distinctly different types and are admirably suited to growth in pots during the summer.

Wagner pots <sup>5</sup> 25 cm. in diameter and 33 cm. in depth were used in these experiments.

## EXPERIMENTS OF THE FIRST SEASON

For the first season the soil was taken from a field of the experiment station called "field G," which for 30 years had been used for comparative studies of potash fertilizers. The soil was a fine sandy loam belonging to the Merrimac series and was a bit heavy for pot experiments.

One lot of soil was taken from the surface of two plots that had been without potash fertilizers for over 30 years, but had been liberally supplied with nitrogen and phosphates. A second lot of soil was mixed from the surface of two plots which had received potash fertilizers annually in addition to the other chemicals—in the one case, muriate of potash; in the other, sulphate. From these two

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<sup>1</sup> Received for publication Oct. 4, 1927; issued December, 1927. Published by permission of the director of the Massachusetts Agricultural Experiment Station.

<sup>2</sup> Credit is due R. L. Coffin for the execution of all details of the pot experiments and to H. D. Haskins and L. S. Walker for the chemical analyses of the crops.

<sup>3</sup> HALL, A. D. *THE BOOK OF THE ROTHAMSTED EXPERIMENTS*. Ed. 2, rev. by E. J. Russell. p. 59, 87. London, J. Murray, 1917.

<sup>4</sup> SEELHORST, C. VON. WIE WIRKT EINE KALIDÜNGUNG AUF DEN WASSERVERBRAUCH DER PFLANZEN UND AUF DEN WASSERGEHALT DER ERDE. *Jour. Landw.* 63: [345]-356. 1915.

<sup>5</sup> LINDSEY, J. B. WAGNER METHOD OF POT CULTURE. U. S. Dept. Agr., Expt. Sta. Rec. 7: 77-87, illus. 1895.

lots of soil there were prepared four different series of pots for comparison, viz: Series A, soil from plots without potash; series B, with potash added to the soil of series A; series C, soil from plots with potash residues; and series D, with potash added to the soil of series C.

By this arrangement, a soil considerably exhausted of its natural potash was compared with a soil containing the residual potash of 30 years' continuous applications of that substance in the fertilizers, and with soluble salts of potash added to both.

Each lot of soil was thoroughly mixed; the amount for each pot was weighed and the chemicals allotted were mixed with it before the pot was filled.

The amounts of fertilizer chemicals used in every pot consisted of 1.5 gm. ammonium nitrate and 3 gm. double superphosphate. The pots which received potash were each allotted 1 gm. of potassium sulphate. These quantities were calculated to be proportional to the amounts per acre used annually on the plots.

Three different quantities of water were used, as follows: A maximum supply calculated to be 50 per cent of the water-holding capacity of the soil, a medium supply of 37.5 per cent, and a minimum supply of 25 per cent.

After the addition of its allotment of water, the gross weight of the pot was thereafter used as the basis for maintaining the supply of soil-moisture approximately uniform from day to day.

Seeds were planted in all the pots on June 7. Germination was completed in a week, and as growth progressed surplus plants were removed until there were left five equidistant plants in each pot.

Early in their growth, both crops began to show the effects of the variations in potash and water supplied them. After about three weeks, the millet plants with the maximum supply of water began to lag behind those with the medium supply, continued to be inferior in size throughout the entire period, and barely surpassed the millet with minimum water supply. Heads appeared first on the millet with the maximum supply of water, while the plants with the minimum supply were the last to show them. Full height of the millet was reached in all pots by August 7, and the plants were harvested on August 30.

The soy beans continued throughout the season to show the effects of the variations in water supply upon the size of the plants. The soy-bean plants that grew in the soils which contained potash ripened normally and were harvested on September 26. The plants in the soil which had received no potash for many years grew abnormally. They remained green and the pods did not fill well. They were not harvested until October 6, when some leaves were yet green and the pods were badly rusted.

When harvested the crop of each pot was put in a cheesecloth sack and hung in the glasshouse, where the bags remained undisturbed, exposed to sun and air, until the chemical analyses could be made.

The air-dry crops were weighed and analyzed. The weights of the crops from duplicate pots agreed well in all the pairs and calculations were based on the combined crops of each pair of pots. Seed and straw were weighed separately with each crop. The millet seed and

straw were remixed and analyzed only as crops. The soy beans from one of each pair of pots of series A and B were analyzed as separated into seeds, pods, and straw because of the marked difference in development between the plants with potash and those without it. The plants from the duplicate pot were remixed and analyzed as a crop.

It was found by several trials that the moisture content of the different crops was quite uniform; consequently, to save labor, subsequent analyses and calculations were all based on the air-dry state.

TABLE 1.—*Weights of air-dry crops and percentages of nitrogen and potash contained in them; first-season experiments*

Crop and series	Water supply	Weight of crop			Nitrogen	Potash
		Seed	Straw	Total		
		Grams	Grams	Grams	Per cent	Per cent
Millet:						
Series A, soil from plots without potash.....	Maximum.....	46	40	86	1.21	0.90
	Medium.....	55	51	106	.94	.72
	Minimum.....	52	50	102	1.08	.56
Series B, potash added to series A.....	Maximum.....	40	42	82	1.05	1.39
	Medium.....	58	64	122	.94	.94
	Minimum.....	44	60	104	.97	.99
Series C, soil from plots with potash residue.....	Maximum.....	44	47	91	.93	1.65
	Medium.....	66	80	146	.84	1.39
	Minimum.....	53	76	129	.81	1.51
Series D, potash added to series C.....	Maximum.....	46	50	96	.82	1.82
	Medium.....	61	76	137	.87	1.72
	Minimum.....	55	74	129	.83	1.66
Soy beans:						
Series A, soil from plots without potash.....	Maximum.....	48	180	228	2.60	.41
	Medium.....	49	159	208	2.66	.47
	Minimum.....	37	107	144	2.90	.55
Series B, potash added to series A.....	Maximum.....	96	191	287	2.62	.61
	Medium.....	82	176	258	2.47	.66
	Minimum.....	55	108	163	2.65	.72
Series C, soil from plots with potash residue.....	Maximum.....	113	222	335	2.09	.91
	Medium.....	99	195	294	2.35	1.08
	Minimum.....	60	115	175	2.56	1.10
Series D, potash added to series C.....	Maximum.....	110	224	334	2.44	1.14
	Medium.....	103	201	304	2.34	1.13
	Minimum.....	60	114	174	2.36	1.28

The outstanding result with millet was the failure of the maximum supply of water to produce the largest plants, although the concentration of potash in them was highest in each series. (Table 1.) The millet in series A, on soil exhausted of potash, yielded by weight more seed than straw. Increased supplies of potash increased the straw proportionally more than the seed.

Soy beans showed a different relation to both potash and water. The maximum supply of water in all series produced the maximum crop, both straw and seed being increased proportionally. The concentration of potash was highest in the crops with least water. Addition of potash to the soil exhausted of potash gave a marked increase in seed, more proportionally than of straw, especially with plenty of water.

TABLE 2.—*Weight of seeds, pods, and straw of soy beans, together with percentages of nitrogen and potash in each, showing the effect of varied supplies of water and of potash*

Item	Weight of crop			Nitrogen in—			Potash in—		
	Seeds	Pods	Straw	Seeds	Pods	Straw	Seeds	Pods	Straw
Series A, without potash:	<i>Grams</i>	<i>Grams</i>	<i>Grams</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Maximum water.....	48	44	136	6.25	1.68	1.61	1.17	0.15	0.23
Medium water.....	49	39	120	6.47	1.60	1.43	1.25	.15	.25
Minimum water.....	37	26	81	6.88	1.57	1.53	1.26	.20	.35
Series B, with potash:									
Maximum water.....	96	57	134	6.25	.68	.87	1.49	.12	.21
Medium water.....	82	50	126	6.16	.63	.81	1.65	.37	.15
Minimum water.....	55	29	79	6.46	.51	.79	1.80	.10	.20

In Table 2 it may be noted that with an abundance of water the addition of potash doubled the weight of seed, increased the weight of pods somewhat, and did not affect the straw, which consisted of the stems and leaves. This in general is the effect with the two series, although as the water supply diminished the addition of potash was less efficient.

The composition of the different parts of the plants is striking in its variations. Seeds are fairly uniform in percentages of nitrogen. The addition of potash produces a somewhat higher percentage of that constituent in series B. Without potash, the pods and straw of series A contain approximately twice as much nitrogen as their corresponding parts in series B. Potash is fairly uniform throughout both series.

The evidence indicates that potash was needed to aid the translocation of nitrogen from stems, leaves, and pods into the seeds, and by helping in this transfer, greatly increased the weight of seeds.

#### EXPERIMENTS OF THE SECOND SEASON

The pot experiments were conducted a second season with some modifications. In addition to the soils from field G, two lots of soil were procured from a field that had been especially responsive to potash in the fertilizer, and has been described in the reports of the experiment station as the "north soil test."<sup>6</sup> One lot of soil was taken from the plot which had received no potash for 30 years and the other lot was from the plot which had received the complete fertilizer, including muriate of potash. Both plots had always been dressed with moderate quantities of nitrate of soda and acid phosphate. By adding potash to each of these soils, four series of soils were prepared: Series A from the plot without potash; series B with potash added to A; series C from the plot with potash; and series D with potash added to C.

Larger amounts of the respective fertilizer chemicals were used this year in all the pots because the quantities employed in the previous season, although proportional to the field applications, were much smaller than experience had shown to be optimum for restricted volumes of soil in pots. Each pot of millet received 6 gm. ammonium nitrate, 7 gm. double superphosphate, and 6 gm. limestone, while

<sup>6</sup> HASKELL, S. B. A THIRTY-YEAR FERTILIZER TEST. Mass. Agr. Expt. Sta. Bul. 212, p. [127]-158, illus. 9122.

the pots of soy beans were given 3 gm. ammonium nitrate, 7 gm. double superphosphate, and 3 gm. limestone. The potash was supplied to the designated pots in 8 gm. sulphate of potash.

The maximum supply of water for the millet was 50 per cent of the water-holding capacity of the soil, while for soy beans it was raised to 60 per cent. The medium supply was 35 per cent for both crops. Millet received as a minimum supply only 20 per cent of the water-holding capacity, but soy beans received 25 per cent, as in the first season. The water-holding capacity of the soils ranged between 52 and 54 per cent, so that they were practically alike in that property.

TABLE 3.—*Weights of air-dry crops and percentages of nitrogen and potash contained in them; second season experiments*

Crop and series	Water supply	Weight of crop			Nitrogen	Potash
		Seed	Straw	Total		
Millet:		Grams	Grams	Grams	Per cent	Per cent
Series A, soil from plots without potash.....	Maximum....	75	64	139	0.88	0.77
	Medium....	68	63	131	.91	.62
	Minimum....	66	56	122	1.60	.49
Series B, potash added to series A.....	Maximum....	91	103	194	.85	1.73
	Medium....	90	107	197	.73	1.89
	Minimum....	92	112	204	1.24	2.10
Series C, soil from plots with potash residue.....	Maximum....	95	97	192	.90	1.60
	Medium....	87	97	184	.80	1.59
	Minimum....	96	95	191	1.37	1.52
Series D, potash added to series C.....	Maximum....	103	111	214	.80	1.87
	Medium....	88	111	199	.77	2.12
	Minimum....	90	107	197	1.38	2.38
Soy beans.						
Series A, soil from plots without potash.....	Maximum....	45	158	203	3.05	.72
	Medium....	41	132	173	2.65	.59
	Minimum....	35	97	132	2.88	.55
Series B, potash added to series A.....	Maximum....	154	257	411	3.10	1.63
	Medium....	103	191	294	2.78	1.80
	Minimum....	74	132	206	2.90	1.82
Series C, soil from plots with potash residue.....	Maximum....	153	240	393	3.16	1.23
	Medium....	101	181	282	2.93	1.43
	Minimum....	67	127	194	2.84	1.28
Series D, potash added to series C.....	Maximum....	152	238	390	2.66	2.20
	Medium....	92	178	270	2.72	2.07
	Minimum....	75	129	204	2.76	1.99
Millet—North soil test series:						
Series A, soil from plot without potash.....	Maximum....	86	72	158	1.03	.80
	Medium....	55	52	107	.95	.36
	Minimum....	59	54	113	1.53	.26
Series B, potash added to series A.....	Maximum....	133	134	267	.90	1.87
	Medium....	82	93	175	.87	2.10
	Minimum....	123	116	239	1.19	2.00
Series C, soil from plots with potash residue.....	Maximum....	92	97	189	.98	.86
	Medium....	105	105	210	1.06	.73
	Minimum....	106	100	206	1.16	.65
Series D, potash added to series C.....	Maximum....	101	111	212	.99	2.23
	Medium....	83	96	179	.87	2.38
	Minimum....	124	117	241	1.04	2.32

Pots were filled and handled as in the first season. Seeds were sown June 20. Germination was prompt and the plants were soon thinned to five in each pot. Growth without potash was again inferior but was proportional to the water supply in the soy beans, while millet showed little difference between maximum and medium supplies.

The millet was harvested on September 12. Plants with the minimum water supply were least matured and numerous heads were imperfect. The pots which received the medium supply of water in series B and D of the north soil test did not yield good parallel weights.

The soy beans grown in series A with little available potash again failed to mature with the rest of the crop. The plants on this soil that received the most water showed many leaves with edges yellow and somewhat curled. The soy beans in all the pots except those just mentioned were harvested 115 days after seeding, but the plants in series A were grown 120 days before cutting, which was performed on October 18.

The crops were dried, weighed, and analyzed as described in the first experiment. The results are shown in Table 3.

The soils of series A that had been without potash fertilizers for years showed a low availability of their natural potash. The percentages of potash in the crops were very low. The soils of series C with their residual potash gave yields about equal to those produced by additions of potash, but with somewhat lower percentages of potash in the crops than were present in those from the addition of potash.

With plenty of available potash, the variations in water supply produced no proportional variations in yields of crops of millet, but soy beans were as much influenced by water supply as by potash. For illustration, in series B with added potash and maximum water supply, the yield of soy beans was double that in series A without the potash; also in series B, the yield with the maximum water supply was double that with the minimum supply. Approximately the same differences in results are to be observed between maximum and minimum water supplies in series C and D.

The effect of potash on seed production in the soy beans was even more striking than in the first season. With an abundance of water the weights of seed with potash were triple the yield without potash.

The effect of potash on the seed production of millet was a repetition of the first season also. Without potash in series A, the yield of seed exceeded the weight of straw. With potash in series B, the straw exceeded the seed. Seed was increased by potash from 20 to 40 per cent, while straw was increased from 60 to 100 per cent.

When the crops from the three series with available potash are compared with those from series A, it may be noted that the crops of millet from the available potash with the least water exceed in weights of seed and of straw the crop in series A with the most water.

Soy beans when similarly compared show a marked increase in seed from the available potash with least water but no increase in the straw. Of these pot experiments it can be said that the presence of potash increased the efficiency of the limited supply of water in developing the plants.

In Table 4 are given the weights of potash actually recovered from the soil in each of the crops. In the first column—series A, without potash for years—it is clearly evident that the availability of the potash is dependent on the water supply, as the amount recovered with the minimum supply of water is much less than that recovered with the maximum supply; while additions of potash to the soil give large increases in the potash found in the crops, undoubtedly due to the greater concentration of the soil solution.

Both variables, potash and water, affected the two crops in these experiments in quite different ways. These different effects are shown most clearly in the second season when nitrogen and phosphorus were in ample supply.

TABLE 4.—*Weights of potash recovered in crops grown with varied supplies of potash and of water*

Crop and season	Water supply	Potash recovered from—			
		Series A (without potash)	Series B (with potash added to A)	Series C (with potash residue)	Series D (with potash added to C)
		Grams	Grams	Grams	Grams
Millet, first season.....	(Maximum.....	0.77	1.14	1.51	1.74
	Medium.....	.76	1.15	2.03	2.35
	(Minimum.....	.57	1.03	1.93	2.14
Soy beans, first season.....	(Maximum.....	.93	1.75	3.05	3.81
	Medium.....	.98	1.70	3.17	3.44
	(Minimum.....	.79	1.17	1.93	2.23
Millet, second season.....	(Maximum.....	1.07	3.35	3.07	4.02
	Medium.....	.81	3.72	2.92	4.22
	(Minimum.....	.60	4.28	2.90	4.69
Soy beans, second season.....	(Maximum.....	1.45	6.70	4.83	8.58
	Medium.....	1.02	5.29	4.03	5.60
	(Minimum.....	.72	3.75	2.49	4.10
Millet, second season (north soil test soil).....	(Maximum.....	.47	4.99	1.62	4.72
	Medium.....	.38	-----	1.53	-----
	(Minimum.....	.29	4.80	1.34	5.59

In Table 3 a comparison of the yields of millet in series A with those in series B shows the gains resulting from the addition of potash to be greatest with the minimum supply of water, while a similar study of the soy-bean crops shows them to have been most responsive to potash with the maximum supply of water.

These differences in their relations to potash and water are corroborated in Table 4 by the weights of potash recovered in the crops in the second season.

The soy beans recovered the most potash when supplied with the maximum quantity of water. Millet recovered the most potash from series B and series D when given the minimum supply of water, with the exception of the north soil test, series B, when it was slightly less than the amount recovered with the maximum water.

A comparison of series C, having potash residue, with series B and series D, both with added potash, shows a marked luxury consumption of that substance. The yields of both seed and total crop in series C, (Table 3) are practically equal to those in series B and D, but the amounts of potash recovered (Table 4) are notably less. Apparently the residual potash of the soil of series C was sufficient for the needs of the soy-bean plants.

The marked increase in the amount of potash recovered by both millet and soy beans from series C in the second season, when nitrogen and phosphoric acid had been supplied more liberally, indicates that the lack of these substance in the first season limited the effect of the potash.

#### SUMMARY

Pot experiments were conducted in which Japanese millet and soy beans were grown with varied amounts of potash and of water supplied to the soil, in order to ascertain the relation of the water supply to the availability of the soil potash and the extent to which additions of soluble potash would overcome the lack of water.

Four series of pots were prepared with relation to potash. Series A contained soil that had received no potash fertilizers for 30 years.

Series B had potash added to the soil of A. Series C contained soil that had received annual applications of potash fertilizers for 30 years. Series D had potash added to the soil of C.

Each series received maximum, medium, and minimum supplies of water, based on the water-holding capacity of the soil. The soils with the least water were more like a naturally dry soil than a soil during a drought because the limited quantities of water were added at short intervals.

The experiments were conducted two seasons. In the first season, the supplies of nitrogen, phosphoric acid, and potash were at the rate used in the field from which the soils were obtained. The supplies of these for the second season were much increased as there were indications that the first year's allowance was too small.

Both millet and soy beans on the soil without potash for years gave yields which varied directly as the supplies of water. The percentages of potash in the crops and the quantities by weight indicated that the soil potash had a low solubility and the amount available for crops was dependent on the supply of water.

The millet crops when supplied with available potash were not much affected by lessened supplies of water. The addition of potash increased the production of straw proportionally more than that of seed.

The soy-bean crops were affected by both potash and water supply and in about the same proportion. Potash increased seed production proportionally more than the yield of straw. On the soil without potash for years, soy beans were slow to mature, and analysis of the seeds, pods, and straw indicated that, with the lack of potash, the nitrogen was not translocated freely from the straw and pods to the seeds.

The plentiful supply of available potash in series B and D in the second season gave an increase in absorption of potash by the crops which was proportionally greater than the increase in plant growth accompanying it.

Available potash with the minimum supply of water enabled millet in the second season to produce 92 gm. of seed and 112 gm. of straw, while without added potash but with abundant water it yielded 75 gm. of seed and 64 gm. of straw. Soy beans under parallel conditions produced 74 gm. of seed with the added potash and 45 gm. without it, but the straw was affected by the water supply.

#### CONCLUSION

The natural potash of the soils used in these pot experiments had a low solubility, and both millet and soy beans obtained their supplies of potash from them in direct proportion to the supplies of water. The addition of potash to the other fertilizers increased the concentration of that substance in the soil solution and the millet was then nearly indifferent to the varied amounts of water supplied to it. Soy beans were about equally affected by both potash and water. For testing the availability of potash in a soil, millet appeared to be a better crop than soy beans because the millet was less affected by variations in the water supply.

The results of the experiment are in accord with the law of the minimum.

# THE INFLUENCE OF POSITION OF CATTLE, AS TO STANDING AND LYING, ON THE RATE OF METABOLISM<sup>1</sup>

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## INTRODUCTION

In 1924 Fries and Kriss published from this Institute a paper<sup>2</sup> which described a new method for the computation of the observed heat production to a standard day as to standing and lying, this method depending on the idea, which was also new, that the true difference in the heat production of standing and lying is a difference in the maintenance requirement of net energy, and, therefore, that this difference may be determined directly from the heat production of standing and lying during fast, since the heat production during fast is the measure of the maintenance requirement of net energy.

The significance of this matter is that in any determination of the effects of the quantity or kind of feed on the heat production of an animal it is necessary consistently to recognize its status as to activity, so that the characteristic effects of the feed on the heat production will be accurately revealed and will not be obscured by the effects of differences in muscular movement.

In this relation it is possible to compensate for differences in time spent in the standing and the lying positions, which constitute a major factor among the voluntary activities of the animal. As to other voluntary activities, the conditions of experimentation, especially as to method of confinement of the subject, serve prominently to affect and to limit their extent and kinds.

Fortunately these other movements, which are not measured in the procedure employed, are not commonly of such extent as to introduce serious confusion into the determination of the heat production as related to the treatment of the animal.

Since the publication of the above-mentioned paper of Fries and Kriss the writers have hoped that this was a solved problem; and it has been the custom in the determination of the net-energy values of feeds at this institute to compute the heat production, as observed, to standard days as to standing and lying, from the time spent by the animal in these positions, and the factor of Fries and Kriss—representing the difference in heat production in these two positions.

As this method has been used, however, for computing the observed heat production in series of experimental days to standard days, as to standing and lying, the agreement of the heat production for the several days in series has not always been much improved, as it presumably would be (unless obscured by experimental errors) if the basis of the computation were satisfactory.

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<sup>1</sup> Received for publication Aug. 8, 1927; issued December, 1927.

<sup>2</sup> FRIES, J. A., and KRISS, M. METABOLISM OF CATTLE DURING STANDING AND LYING. *Amer. Jour. Physiol.* 71: 60-83. 1924.

On this account the writers have been obliged to consider this a continuing problem, and after having been on the lookout for some time for a new avenue of approach, they are now able to make a further contribution on the subject, revealing the problem in a new light, and having the effect very extensively to increase the magnitude of the factor representing the difference in energy cost of standing and lying.

The quantitative determination of the comparative energy metabolism during standing and lying, either by direct or indirect calorimetry, presents many difficulties, and the results obtained by different investigators vary widely, in accord with differences in fundamental conceptions involved, and under the influence of different conditions of experimentation, especially as to size of animal and plane of nutrition. Thus Hagemann,<sup>3</sup> in two experiments with steers, reported increases of 28 per cent and 30 per cent in the total heat production of standing as compared with lying. Dahm,<sup>4</sup> working in Zuntz's laboratory, and by Zuntz's method, found an increase of only 8 per cent in the respiratory excretion of carbon dioxide by a young bull, when standing as compared with lying. Klein<sup>5</sup> studied the respiratory exchange of a steer, during standing and lying, by means of a tracheal cannula, and found an increase of 20.7 per cent in the heat production of standing as compared with lying. Armsby and Fries<sup>6</sup> calculated that in 37 published experiments with steers the increase in the directly determined heat production during standing as compared with lying varied from a minimum of 28.3 per cent to a maximum of 64.5 per cent, averaging 41.4 per cent; and they stated that a considerable number of other experiments, unpublished at that time, gave similar results. Armsby and Fries also reported results of six experiments with a steer in which the carbon dioxide and water vapor, as well as the heat produced, were determined separately for intervals of standing and lying, and showed increases in carbon dioxide elimination during standing as compared with lying varying from 20.4 to 35.1 per cent, with corresponding increases in heat production varying between 32.3 and 40.0 per cent.

In all of the experiments referred to above the animals received feed.

Recently Fries and Kriss<sup>7</sup> pointed out certain instrumental errors and imperfections in the method used by Armsby and Fries to separate the directly measured heat production between intervals of standing and lying, especially in feeding periods, and by an indirect computation concluded that a fasting cow, weighing 400 kgm., gave off while standing 26.3 Calories more per hour than while lying, this increase being equal to 9.8 per cent of the total heat production.

This figure was derived from observations on a single cow, but under conditions regarded at the time as unusually favorable. In the light of the present paper, however, which is based upon much

<sup>3</sup> HAGEMANN, O. DAS RESPIRATIONS-CALORIMETER IN BONN UND EINIGE UNTERSUCHUNGEN MIT DEMSELBEN BEI ZWEI RINDERN UND EINEM PFERDE. *Landw. Jahrb.* 41 (Ergänzbd. 1): 61, 128. 1911.

<sup>4</sup> DAHM, K. DIE BEDEUTUNG DES MECHANISCHEN THEILS DER VERDAUUNGSARBEIT FÜR DEN STOFFWECHSEL DES RINDES. *Biochem. Ztschr.* 28: 457-503. 1910.

<sup>5</sup> HEIDE, R. VON DER KLEIN, and ZUNTZ, N. RESPIRATIONS UND STOFFWECHSELVERSUCHE AM RINDE ÜBER DEN NÄHRWERT DER KARTOFFELSCHLEMPPE UND IHRER AUSGANGSMATERIALIEN. *Landw. Jahrb.* 44: [765]-832. 1913.

<sup>6</sup> ARMSBY, H. P., and FRIES, J. A. INFLUENCE OF STANDING OR LYING UPON THE METABOLISM OF CATTLE. *Amer. Jour. Physiol.* 31: 245-253. 1913.

<sup>7</sup> FRIES, J. A., and KRISS, M. *Op. cit.*

more extensive data, and improved technic and computations, the problem presented by the instrumental lag, in the study by Fries and Kriss, appears not to have been successfully handled; and it seems imperative, therefore, to reconsider the whole subject.

#### CONDITIONS OF EXPERIMENTATION

The subjects of this investigation were two Aberdeen Angus steers designated as Nos. 36 and 47, their live weights being 468 and 479 kgm., respectively.

In the spring of 1927 each of these steers was subjected to a fast, steer No. 36 for a period of 7 days, and steer No. 47 for a period of 6 days. During the last four days of these fasting periods the animals were subjected to continuous respiration calorimetric study.

For the purpose of this investigation, in addition to the usual heat measurement, samples of the air coming from the calorimeter chamber were taken at intervals of 15 minutes, during two of the four calorimeter days, for the determination of  $\text{CO}_2$  production. In case of steer No. 47 such samples were taken during the second and third of the calorimeter days—these being the fourth and fifth days of fast. With steer No. 36 such samples were taken during the last two calorimeter days—the sixth and seventh days of fast. Carbon dioxide was determined in these air samples by means of a Sondén apparatus.

The rate of ventilation was practically constant during these experiments. Throughout their course record was kept of the time of all changes of position of the animals, as to standing and lying. Instead of considering the results, as usual, for experimental days of 24 hours, however, the data involved in this study are discussed in 12-hour subperiods.

The animals were allowed to change position at will, except during one interval of three and a half hours in the first half of the last day (subperiod 7) in the case of steer No. 36. During this interval the steer was prevented from lying down by means of a chain, purposely connected across the calorimeter stall beneath the animal's body, but sufficiently low so that the animal could not rest upon it. This was done to make possible the collection of air samples certainly and accurately representative of the standing position, since both animals changed position rather frequently and spent much more time lying than standing. This procedure satisfactorily served the intended purpose. The animal was rather quiet, and made no apparent effort to lie down while the chain was connected.

#### DETERMINATION OF THE CARBON DIOXIDE PRODUCTION FOR STANDING AND LYING

It was realized, from the beginning of this study, that samples of air drawn from the chamber soon after the animal had changed position would not be accurately representative of the new position, on account of instrumental lag. The air capacity of the chamber, with the animal inside, was calculated to be approximately 11,000 liters, and the passage of air through the chamber was at the rate of 450 liters per minute. At this rate of ventilation, therefore, about 25 minutes were required to pump through the chamber a volume of air equal to the chamber capacity. The all-important question as to

how much more than 25 minutes was required for the air in the chamber to reach a composition representative of the position of the animal was left to be answered by the experimental data.

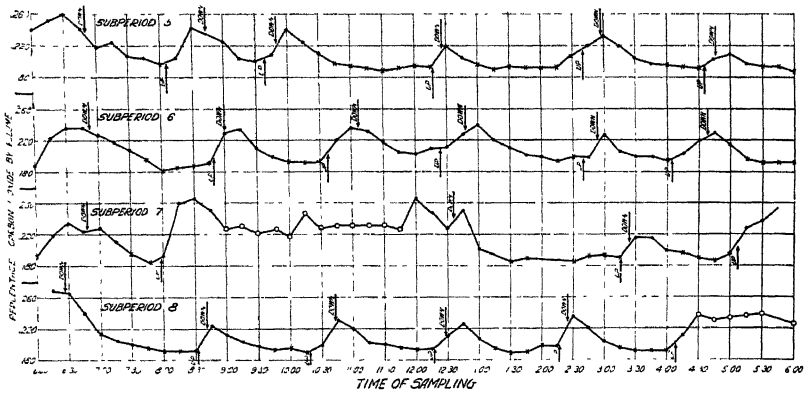


FIG. 1.—Influence of standing and lying on the carbon dioxide content of the air coming from the respiration chamber, steer No. 36. (Stars represent lying position, circles, the standing position)

It was anticipated that the analysis of the air samples taken at frequent and regular intervals (15 minutes) would make possible the tracing of the instrumental lag, and the determination of the time of

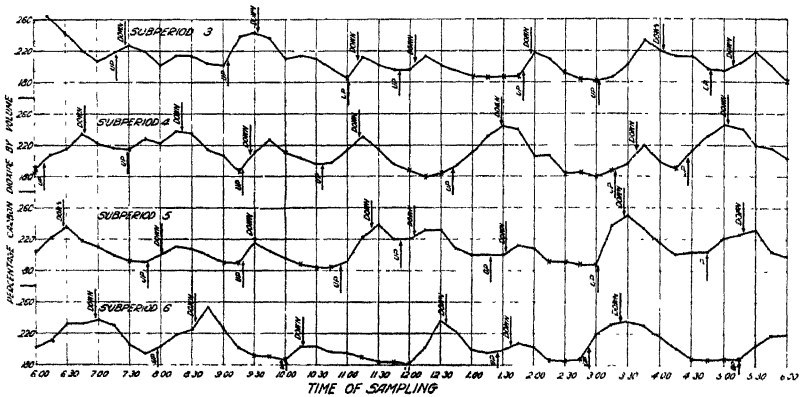


FIG. 2.—Influence of standing and lying on the carbon dioxide content of the air coming from the respiration chamber; steer No. 47. (Stars represent lying position)

operation necessary for the reduction of this lag to a negligible factor. For the accomplishment of this purpose  $\text{CO}_2$  was determined in the 15-minute samples, as indicated in Table 1, and the results were plotted. (Figs. 1 and 2.)

STEER NO. 47

[illegible]

TABLE 1.—Carbon dioxide content of outgoing air as affected by the position of fasting steers; experiment No. 238—Continued

STEER NO. 47—Continued

Date	Time of sampling	Position of animal, and time of change of position	Carbon dioxide by volume	Date	Time of sampling	Position of animal, and time of change of position	Carbon dioxide by volume
Subperiod 5	<i>p. m.</i>	<i>p. m.</i>	<i>Per cent</i>	Subperiod 6	<i>p. m.</i>	<i>p. m.</i>	<i>Per cent</i>
	4.00	Down 3.26	0.216		11.30	Down 10.16	0.184
	4.15	do	.201		11.45	do	.184
	4.30	do	.204				
	4.45	Up, 4.45	.204				
	5.00	do	.221				
	5.15	do	.226				
	5.30	Down, 5.19	.232				
	5.45	do	.204				
	6.00	do	.199				
Subperiod 6	6.15	Up, 6.07	.212				
	6.30	do	.232				
	6.45	do	.232				
	7.00	Down, 6.56	.238				
	7.15	do	.229				
	7.30	do	.206				
	7.45	do	.194				
	8.00	Up, 7.56	.202				
	8.15	do	.219				
	8.30	do	.225				
	8.45	Down, 8.33	.254				
	9.00	do	.225				
	9.15	do	.202				
	9.30	do	.192				
	9.45	do	.191				
	10.00	do	.186				
	10.15	Up, 10.01	.204				
	10.30	Down, 10.16	.204				
	10.45	do	.196				
	11.00	do	.196				
	11.15	do	.190				

STEER NO. 36

May 5, 1927, subperiod 5	<i>a. m.</i>	<i>a. m.</i>		May 5, 1927, subperiod 5	<i>p. m.</i>	<i>p. m.</i>	
	6.00	Up, 5.17	0.240		2.15	Down 12.26	0.192
	6.15	do	.252		2.30	do	.209
	6.30	do	.260		2.45	Up, 2.41	.219
	6.45	do	.238		3.00	Down, 2.58	.233
	7.00	Down, 6.50	.217		3.15	do	.220
	7.15	do	.224		3.30	do	.204
	7.30	do	.206		3.45	do	.198
	7.45	do	.205		4.00	do	.196
	8.00	do	.196		4.15	do	.194
	8.15	Up, 8.06	.204		4.30	do	.193
	8.30	do	.244		4.45	Up, 4.35	.204
	8.45	Down, 8.43			5.00	Down, 4.46	.210
	9.00	do	.226		5.15	do	.198
	9.15	do	.205		5.30	do	.194
	9.30	do	.199		5.45	do	.195
	9.45	Up, 9.39	.208		6.00	do	.188
	10.00	Down, 9.50	.242		6.15	Up, 6.08	.224
	10.15	do	.224		6.30	do	.237
	10.30	do	.210		6.45	do	.235
	10.45	do	.197		7.00	Down, 6.52	.227
	11.00	do	.195		7.15	do	.219
	11.15	do	.192		7.30	do	.207
	11.30	do	.187		7.45	do	.196
	11.45	do	.192		8.00	do	.182
					8.15	do	.186
					8.30	do	.189
					8.45	do	.191
	Noon						
	12.00	do	.194				
	<i>p. m.</i>						
	12.15	do	.192				
		<i>p. m.</i>					
		Up, 12.18					
	12.30	Down, 12.26	.219				
	12.45	do	.204				
	1.00	do	.196				
	1.15	do	.190				
	1.30	do	.194				
	1.45	do	.192				
	2.00	do	.192				

TABLE 1.—Carbon dioxide content of outgoing air as affected by the position of fasting steers; experiment No. 238—Continued

STEER No. 36—Continued

Date	Time of sampling	Position of animal, and time of change of position	Carbon dioxide by volume	Date	Time of sampling	Position of animal, and time of change of position	Carbon dioxide by volume
May 6, 1927	<i>Midnight</i>	<i>p. m.</i>	<i>Per cent</i>	Subperiod 7	<i>p. m.</i>	<i>p. m.</i>	<i>Per cent</i>
	12.00	Down, 11.06	0.204		3.15	Down, 12.36	0.192
	12.15	do	.211			Up, 3.16	
					3.30	Down, 3.24	.218
	<i>a. m.</i>	<i>a. m.</i>			3.45	do	.216
	12.30	Up, 12.23	.212		4.00	do	.200
	12.45	do	.229		4.15	do	.197
	1.00	Down, 12.47	.240		4.30	do	.191
	1.15	do	.221		4.45	do	.187
	1.30	do	.212	Subperiod 8	5.00	do	.195
	1.45	do	.203		5.15	Up, 5.07	.228
	2.00	do	.200		5.30	do	.236
	2.15	do	.194		5.48	do	.254
	2.30	do	.201		6.15	do	.269
	2.45	Up, 2.40	.198		6.30	Down, 6.27	.266
	3.00	Down, 2.54	.228		6.45	do	.241
	3.15	do	.206		7.00	do	.214
	3.30	do	.199		7.15	do	.206
	3.45	do	.200		7.30	do	.201
Subperiod 7	4.00	do	.194		7.45	do	.195
	4.15	Up, 4.04	.204		8.00	do	.193
	4.30	do	.220		8.15	do	.193
	4.45	Down, 4.37	.230		8.30	do	.192
	5.00	do	.214			Up, 8.32	
	5.15	do	.196		8.45	Down, 8.40	.224
	5.30	do	.192		9.00	do	.214
	5.45	do	.193		9.15	do	.204
	6.00	do	.193		9.30	do	.198
	6.15	Up, 6.02	.219		9.45	do	.194
	6.30	do	.236		10.00	do	.195
	6.45	do	.225		10.15	do	.190
	7.00	Down, 6.48	.229		10.30	Up, 10.19	.199
	7.15	do	.212		10.45	Down, 10.43	.231
	7.30	do	.197		11.00	do	.221
	7.45	do	.185		11.15	do	.203
	8.00	Up, 7.58	.194		11.30	do	.201
	8.15	do	.260		11.45	do	.197
	8.30	do	.266	May 7, 1927	<i>Midnight</i>		
	8.45	do	.252		12.00	do	.194
	9.00	do	.227				
	9.15	do	.231		<i>a. m.</i>		
	9.30	do	.222		12.15	do	.195
	9.45	do	.227				
	10.00	do	.218			<i>a. m.</i>	
	10.15	do	.249			Up, 12.17	
	10.30	do	.228		12.30	Down, 12.28	.212
	10.45	do	.232		12.45	do	.226
	11.00	do	.232		1.00	do	.208
	11.15	do	.232		1.15	do	.195
	11.30	do	.231		1.30	do	.190
	11.45	do	.226		1.45	do	.191
					2.00	do	.199
	<i>Noon</i>				2.15	do	.198
	12.00	do	.266			Up, 2.17	
					2.30	Down, 2.26	.236
	<i>p. m.</i>				2.45	do	.221
	12.15	do	.249		3.00	do	.204
	12.30	do	.228		3.15	do	.197
		<i>p. m.</i>			3.30	do	.193
	12.45	Down, 12.36	.252		3.45	do	.192
	1.00	do	.202		4.00	do	.192
	1.15	do			4.15	Up, 4.08	.213
	1.30	do	.186		4.30	do	.237
	1.45	do	.190		4.45	do	.230
	2.00	do			5.00	do	.234
	2.15	do			5.15	do	.236
	2.30	do	.186		5.30	do	.238
	2.45	do	.194		5.45	do	
	3.00	do	.194		6.00	do	.226

The several graphs indicate clearly the course of metabolism during standing and lying as affected by the instrumental lag. They show that after a change of position about an hour was required for the CO<sub>2</sub> of the outgoing air to reach essentially a constant. Such

parts of the graphs as appear to be practically beyond the influence of the instrumental lag have been designated by special marks, those representing the lying position being starred, while those representing the standing position are marked by circles. The corresponding percentages of  $\text{CO}_2$  for standing and lying have been averaged, and are presented in Tables 2 and 3.

Since in six of the eight 12-hour subperiods the animal did not stand for sufficiently long intervals to permit the direct determination of the  $\text{CO}_2$  production for standing, this was obtained indirectly through a computation by difference. This computation, in general terms, consisted in subtracting the  $\text{CO}_2$  production as determined for the entire time spent in the lying position from the total  $\text{CO}_2$  produced during the 12-hour subperiod, and relating the remainder to the time spent by the animal in the standing position during the same subperiod. This computation, the results of which are presented in Table 4, was performed as indicated below:

(a) The total  $\text{CO}_2$  in the outcoming air was obtained by multiplying the average percentage of  $\text{CO}_2$  of all samples analyzed, by means of the Sondén apparatus, by the quantity of the total ventilation.

(b) To the total  $\text{CO}_2$  obtained in (a) a residual correction, derived from a special 15-minute continuous aspirator sample of the outcoming air, was applied, to correct for the difference in composition of the air in the respiration chamber at the end as compared with the beginning of a 12-hour subperiod.

(c) From the total corrected  $\text{CO}_2$  in the outcoming air was subtracted the total  $\text{CO}_2$  of the ingoing air, as determined gravimetrically in a continuous sample. The remainder is the total  $\text{CO}_2$  produced by the animal.

(d) The total outcoming air during lying was computed from the total ventilation, in proportion to the time spent in the lying position.

(e) The liters of  $\text{CO}_2$  in the outcoming air during lying were obtained by multiplying the average per cent of  $\text{CO}_2$  for lying, as in Table 2, by the volume of outcoming air during lying, as obtained in (d).

(f) The liters of  $\text{CO}_2$  in the outcoming air during standing were computed by subtracting the liters of  $\text{CO}_2$  in the outcoming air during lying, as obtained in (e), from the total  $\text{CO}_2$  in the outcoming air, as obtained in (b).

(g) The quantities of  $\text{CO}_2$  in the ingoing air during lying and standing, respectively, were computed from the total  $\text{CO}_2$  of the ingoing air in proportion to the time spent in the lying and the standing positions.

(h) The  $\text{CO}_2$  produced during lying and standing, respectively, was obtained by subtracting from the  $\text{CO}_2$  in the outcoming air for lying and standing (e and f) the corresponding quantities of  $\text{CO}_2$  in the ingoing air.

(i) The  $\text{CO}_2$  produced per hour, during lying and standing, respectively, was calculated from the corresponding totals, as obtained in (h), and the durations of time of lying and standing represented.

The data obtained in (f), (h), and (i), for standing, however, include the effect of the effort of rising and of lying down. The  $\text{CO}_2$  produced during standing, excluding this effect of the effort of rising and lying down, was obtained by the following calculations:

(j) The ratio of heat to  $\text{CO}_2$  was computed by dividing the number of Calories of observed heat production by the liters of  $\text{CO}_2$  produced, as obtained in (c).

(*k*) The energy required for rising and lying down was computed by the use of Klein's<sup>8</sup> factor of 9.7 Calories per act of rising and lying down, together, by a steer weighing 550 kgm.; this factor, modified directly according to the weight of the animal, being multiplied by the number of times the animal arose and lay down again.

(*l*) The CO<sub>2</sub> required for rising and lying down was obtained by dividing the Calories required for rising and lying down by the heat-CO<sub>2</sub> ratio.

(*m*) The CO<sub>2</sub> produced during standing, excluding the effect of the act of rising and lying down, was computed by subtracting the CO<sub>2</sub> required for rising and lying down from the CO<sub>2</sub> produced during standing, as obtained in (*h*). The same was also computed, per hour, as in (*i*).

(*n*) The CO<sub>2</sub> produced during standing by steer No. 36 in subperiods 7 and 8 was also computed directly by first multiplying the average percentage of CO<sub>2</sub> in the outcoming air during standing, as presented in Table 3, by the volume of the outcoming air during standing, and then subtracting from the product the corresponding quantity of CO<sub>2</sub> of the ingoing air.

#### DISCUSSION OF RESULTS

In general the results obtained with steer No. 36 may be regarded as more satisfactory than those with steer No. 47, principally because the former changed position less frequently than the latter. The number of intervals of time spent by steer No. 36 in the lying position during a 12-hour subperiod varied from 3 to 6, while in the case of steer No. 47 the number varied from 7 to 8. The intervals spent in one position, therefore, were longer, in most cases, with steer No. 36 than with steer No. 47. This fact afforded a relatively better basis for the determination of the CO<sub>2</sub> production during lying with steer No. 36, as can be seen both in Figures 1 and 2 and in Table 2. It also provided, with this animal, a basis for the direct determination of the CO<sub>2</sub> production during standing, in subperiods 7 and 8 (Table 3), whereas such a determination was not possible with steer No. 47 in any of the subperiods.

The data presented in Table 2 represent the percentages of CO<sub>2</sub> in the outcoming air during lying, with the factor of instrumental lag presumably excluded. If there were also a physiological lag in the adjustment of metabolism to changes of position, this would be indistinguishable, by the method of experimentation employed, from the instrumental lag; and, for the present purpose, a procedure adequate to the elimination of the experimental lag, as observed, may be considered to cover the instrumental lag and a conceivable physiological lag, as well. In this relation, however, the data of Table 2 have a special significance, since they represent the metabolism of the animal adjusted to the lying position.

The average percentages CO<sub>2</sub> in the outcoming air during lying are, with steer No. 36—0.193, 0.192, 0.191, and 0.194, in subperiods 5, 6, 7, and 8, respectively. With steer No. 47 the corresponding values are 0.186, 0.185, 0.189, and 0.187, for subperiods 3, 4, 5, and 6, respectively. There is, therefore, good agreement between the subperiods with both animals. This agreement is significant, because the rate of ventilation was practically constant throughout the experiment.

<sup>8</sup>HEIDE, R. VON DER KLEIN, and ZUNTZ, N. Op. cit.

TABLE 2.—Percentages of carbon dioxide in outcoming air representing metabolism during lying; experiment No. 238

Period 11, steer No. 47, fasting			Period 12, steer No. 36, fasting		
Subperiod	Time of sampling	Percent-age CO <sub>2</sub>	Subperiod	Time of sampling	Percent-age CO <sub>2</sub>
No. 3.....	<i>a. m.</i> 11.00	0.185	No. 5.....	<i>a. m.</i> 8.00	0.196
				10.45	.197
	<i>p. m.</i> 1.00	.188		11.00	.195
	1.15	.186		11.15	.192
	1.30	.187		11.30	.187
	1.45	.187		11.45	.192
	2.30	.193		Noon 12.00	.194
	2.45	.184		<i>p. m.</i> 12.15	.192
	3.00	.182		1.00	.196
	6.00	.182		1.15	.190
Average.....		.186	Average.....		.193
No. 4.....	<i>p. m.</i> 9.15	.186	No. 6.....	<i>p. m.</i> 6.00	.188
	Midnight 12.00	.189		7.45	.196
	<i>a. m.</i> 12.15	.180		8.00	.182
	12.30	.184		8.15	.186
	2.30	.183		8.30	.189
	2.45	.186		8.45	.191
	3.00	.179		10.00	.194
	3.15	.189		10.15	.193
	4.15	.190		10.30	.193
Average.....		.185	Average.....		.192
No. 5.....	<i>a. m.</i> 7.30	.193	No. 7.....	<i>a. m.</i> 6.00	.193
	7.45	.191		7.30	.197
	9.00	.190		7.45	.185
	9.15	.190		<i>p. m.</i> 1.30	.186
	10.15	.189		1.45	.190
	10.30	.183		2.30	.186
	10.45	.183		2.45	.194
	<i>p. m.</i> 2.15	.193		3.00	.194
	2.30	.191		3.15	.192
	2.45	.189		4.15	.197
Average.....		.189	Average.....		.191
No. 6.....	<i>p. m.</i> 9.30	.192	No. 8.....	<i>p. m.</i> 7.45	.195
	9.45	.191		8.00	.193
	10.00	.186		8.15	.193
	11.15	.190		8.30	.192
	11.30	.184		10.45	.194
	11.45	.184		10.00	.195
				10.15	.190
				11.45	.197

TABLE 2.—Percentages of carbon dioxide in outcoming air representing metabolism during lying; experiment No. 238—Continued

Period 11, steer No. 47, fasting			Period 12, steer No. 36, fasting		
Subperiod	Time of sampling	Percent-age CO <sub>2</sub>	Subperiod	Time of sampling	Percent-age CO <sub>2</sub>
No. 6-----	<i>Midnight</i> 12. 00	0. 181	No. 8-----	<i>Midnight</i> 12. 00	0. 194
	<i>a. m.</i> 2. 15	. 186		<i>a. m.</i> 12. 15	. 195
	2. 30	. 186		1. 15	. 195
	2. 45	. 187		1. 30	. 190
	4. 30	. 188		1. 45	. 191
	4. 45	. 187		2. 00	. 199
	5. 00	. 188		2. 15	. 198
	5. 15	. 189		3. 15	. 197
				3. 30	. 193
Average-----		. 187	Average-----	3. 45	. 192
				4. 00	. 192
					. 194

TABLE 3.—Percentages of carbon dioxide in outcoming air representing metabolism during standing; experiment No. 238

## PERIOD 12, STEER NO. 36, FASTING

Subperiod	Time of sampling	Percent-age CO <sub>2</sub>	Subperiod	Time of sampling	Percent-age CO <sub>2</sub>
No. 7-----	<i>a. m.</i> 9. 00	0. 227	No. 8-----	<i>a. m.</i> 4. 30	0. 237
	9. 15	. 231		4. 45	. 230
	9. 30	. 222		5. 00	. 234
	9. 45	. 227		5. 15	. 236
	10. 00	. 218		5. 30	. 238
	10. 15	. 249		6. 00	. 226
	10. 30	. 228			
	10. 45	. 232			
	11. 00	. 232			
	11. 15	. 232			
	11. 30	. 231			
	11. 45	. 226			
Average-----		. 230	Average-----		. 234

Table 3 gives the percentages of CO<sub>2</sub> in the outcoming air representing the metabolism of the animal while standing, and also exclusive of the influence of the experimental lag. In subperiod 7 the data represent enforced standing, while those of subperiod 8 represent voluntary standing. These data do not exhibit any significant difference between the enforced and the voluntary standing, the average percentage of CO<sub>2</sub> in subperiod 7 being 0.230, and in subperiod 8, —0.234.

The values for CO<sub>2</sub> production per hour, during lying, which appear in Table 4, and which were derived from the data of Table 2, exhibit a very good agreement between the subperiods as well as between the two animals. These values for steer No. 47 in the four subperiods here considered are 42.0, 41.5, 42.5, and 42.1 liters, respectively, averaging 42.0 liters, while for steer No. 36 the corresponding values are 43.3, 43.0, 43.1, and 43.8 liters, averaging 43.3 liters. Steer No. 36 appears to have produced slightly more CO<sub>2</sub> per hour while lying than did steer No. 47. It should be borne in mind that the above values represent the metabolism of the animal during lying, exclusive of the influence of the experimental lag.

TABLE 4.—Carbon dioxide production during standing and lying; experiment No. 238  
PERIOD 11, STEER NO. 47, FASTING

Item	Subperiod			
	3	4	5	6
Total ventilation.....liters.....	324, 113.5	324, 399.2	322, 125.7	322, 343.1
CO <sub>2</sub> in outgoing air.....per cent.....	0.209	0.211	0.209	0.207
CO <sub>2</sub> in outgoing air.....liters.....	677.4	684.5	673.2	667.3
Residual correction.....do.....	-6.5	+1.6	-0.4	+3.2
Total CO <sub>2</sub> in outgoing air, corrected.....do.....	670.9	686.1	672.8	670.5
Total CO <sub>2</sub> in ingoing air.....do.....	98.7	102.7	99.0	97.1
Total CO <sub>2</sub> produced.....do.....	572.2	583.4	573.8	573.4
Time spent lying.....minutes.....	552	482	562	512
Time spent standing.....do.....	168	238	158	208
Outcoming air during lying.....liters.....	248, 486.7	217, 167.2	251, 437.0	229, 221.8
CO <sub>2</sub> in outgoing air during lying.....per cent.....	0.186	0.185	0.189	0.187
CO <sub>2</sub> in outgoing air during lying.....liters.....	462.2	401.8	475.2	428.6
CO <sub>2</sub> in outgoing air during standing.....do.....	208.7	284.3	197.6	241.9
CO <sub>2</sub> in ingoing air during lying.....do.....	75.7	68.8	77.3	69.0
CO <sub>2</sub> in ingoing air during standing.....do.....	23.0	33.9	21.7	28.1
CO <sub>2</sub> produced during lying.....do.....	386.5	333.0	397.9	359.6
CO <sub>2</sub> produced during standing.....do.....	185.7	250.4	175.9	213.8
CO <sub>2</sub> produced during lying, per hour.....do.....	42.0	41.5	42.5	42.1
CO <sub>2</sub> produced during standing, per hour.....do.....	66.3	63.1	66.8	61.7
Total heat production.....Calories.....	3,703.4	3,843.4	3,628.3	3,732.9
Heat: CO <sub>2</sub> .....do.....	6.47	6.59	6.32	6.51
Energy required for rising and lying down.....Calories.....	63.8	59.5	68.0	59.5
CO <sub>2</sub> required for rising and lying down.....liters.....	9.9	9.0	10.8	9.1
CO <sub>2</sub> produced during standing, excluding act of rising and lying down.....liters.....	173.8	241.4	165.1	204.7
CO <sub>2</sub> produced during standing, excluding act of rising and lying down, per hour.....liters.....	62.8	60.9	62.7	59.0

PERIOD 12, STEER NO. 36, FASTING

Item	Subperiod			
	5	6	7	8
Total ventilation.....liters.....	324, 129.4	324, 392.4	323, 776.3	324, 486.2
CO <sub>2</sub> in outgoing air.....per cent.....	0.208	0.209	0.220	0.212
CO <sub>2</sub> in outgoing air.....liters.....	674.2	678.0	710.4	687.9
Residual correction.....do.....	-5.1	+0.2	+6.6	-3.4
Total CO <sub>2</sub> in outgoing air, corrected.....do.....	669.1	678.2	717.0	684.5
Total CO <sub>2</sub> in ingoing air.....do.....	106.0	106.7	101.2	103.6
Total CO <sub>2</sub> produced.....do.....	563.1	571.5	615.8	580.9
Time spent lying.....minutes.....	586	560	335	529
Time spent standing.....do.....	134	160	385	191
Outcoming air during lying.....liters.....	263, 805.3	252, 305.2	150, 615.9	238, 407.2
CO <sub>2</sub> in outgoing air during lying.....per cent.....	0.193	0.192	0.191	0.194
CO <sub>2</sub> in outgoing air during lying.....liters.....	509.1	484.4	287.7	462.5
CO <sub>2</sub> in outgoing air during standing.....do.....	160.0	193.8	429.3	222.0
CO <sub>2</sub> in ingoing air during lying.....do.....	86.3	83.0	47.1	76.1
CO <sub>2</sub> in ingoing air during standing.....do.....	19.7	23.7	54.1	27.5
CO <sub>2</sub> produced during lying.....do.....	422.8	401.4	240.6	386.4
CO <sub>2</sub> produced during standing.....do.....	140.3	170.1	375.2	194.5
CO <sub>2</sub> produced during lying, per hour.....do.....	43.3	43.0	43.1	43.8
CO <sub>2</sub> produced during standing, per hour.....do.....	62.8	63.8	58.5	61.1
Total heat production.....Calories.....	3,730.4	3,779.9	4,081.8	3,672.0
Heat: CO <sub>2</sub> .....do.....	6.62	6.61	6.63	6.32
Energy required for rising and lying down.....Calories.....	49.8	49.8	29.1	11.5
CO <sub>2</sub> required for rising and lying down.....liters.....	7.5	7.5	4.4	6.6
CO <sub>2</sub> produced during standing, excluding act of rising and lying down.....liters.....	132.8	162.6	370.8	187.9
CO <sub>2</sub> produced per hour during standing, excluding act of rising and lying down.....liters.....	59.5	61.0	57.8	59.0
CO <sub>2</sub> produced per hour during standing, excluding act of rising and lying down, directly determined.....liters.....			53.6	54.6

<sup>a</sup> 8.3 Calories per act of rising and lying down.<sup>b</sup> Corrected for man in chamber by 1.9 l. CO<sub>2</sub>.

Two sets of figures for each animal are the results of the computation, by difference, of the CO<sub>2</sub> production during standing. The first set of figures result directly from subtracting the CO<sub>2</sub> production during lying from the total CO<sub>2</sub> produced, and comprise therefore in addition to the CO<sub>2</sub> of standing, the CO<sub>2</sub> produced as a result of the effort of rising and of lying down. Expressed as volumes per hour these are 66.3, 63.1, 66.8, and 61.7 liters, respectively, in the four

subperiods with steer No. 47; and 62.8, 63.8, 58.5, and 61.1 liters in the four subperiods with steer No. 36.

When corrected for the estimated  $\text{CO}_2$  required for rising and lying down the above figures become 62.8, 60.9, 62.7, and 59.0 liters, averaging 61.4 liters, for steer No. 47; and 59.5, 61.0, 57.8, and 59.0 liters, averaging 59.3 liters, for steer No. 36. These corrected values represent the metabolism of the animal during standing, excluding the act of rising and lying down, but including the possible physiological effect of adjustment of the metabolism to the changes in position and the various activities of the animal while standing during the experiment. Aside from the uncertainty of the validity of the factor upon which the corrections for rising and lying down were based, the values for  $\text{CO}_2$  production during standing, computed by the difference method, have, therefore, a different significance from such values as determined directly.

The direct determination of the  $\text{CO}_2$  production during standing was possible only in two subperiods with steer No. 36, namely, subperiods 7 and 8. The values obtained are based on two intervals of standing, the one covering 4 hours and 38 minutes (from 7.58 a. m. to 12.36 p. m.), in subperiod 7, during which the animal was forced to stand; and the other covering only 1 hour and 52 minutes (from 4.08 a. m. to 6.00 a. m.), in subperiod 8, representing voluntary standing. In subperiod 7 the animal produced 53.6 liters of  $\text{CO}_2$  per hour while standing, and in subperiod 8, —54.6 liters per hour. The difference between these values is small, especially in consideration of the differences in the unrecorded activities of the animal while standing, and therefore indicates that there was no material difference in the metabolism of the forced and the voluntary standing.

The average of the two directly determined values for  $\text{CO}_2$  production during standing is 54.1 liters per hour, and can be compared with the average  $\text{CO}_2$  production by the same animal while lying, which is 43.3 liters; both being determined in such manner as to exclude the influence of instrumental and possible physiological lag. The difference is 10.8 liters per hour. In other words, the steer, weighing 468 kgm., produced 10.8 liters of  $\text{CO}_2$  per hour more during standing than during lying. Expressed in relation to 100 kgm. live weight, the increase in  $\text{CO}_2$  production due to the effort of standing is 2.31 liters per hour.

The computation of this factor from the basis of the live weight of the animal on which it was determined, to another live-weight basis, may be made in accord with the two-thirds power of the live weight, in consideration of the fact that the basal metabolism varies with the surface area (and disregarding the difference between the basal metabolism, which is measured by the fasting and resting heat production, and the maintenance requirement of net energy, which is measured as the 24-hour fasting metabolism with normal activity), or it may be computed directly in accord with the live weight, as above. The difference is not extensive.

The increase in heat production of standing as compared with lying may be computed by multiplying the corresponding increase in  $\text{CO}_2$  by the average heat- $\text{CO}_2$  ratio obtained with the same animal,

on the assumption that this ratio, during fast, is the same for standing and lying.

The heat-CO<sub>2</sub> ratios  $\left(\frac{\text{Calories of heat}}{\text{liters of CO}_2}\right)$  obtained with steer No. 36 in the four subperiods are 6.62, 6.61, 6.63, and 6.32: The writers are unable to explain the divergence of the value for subperiod 8 from the three other values. The average of the first three is 6.62. If the fourth value is included the average is 6.55.

For the purpose of computing the difference in heat production between standing and lying it makes very little difference (less than 1 Calorie per head per hour) which of the above average heat-CO<sub>2</sub> ratios is used. On the basis of the average ratio (6.55) for all four subperiods with steer No. 36 the increase in heat production for standing as compared with lying was 70.7 Calories ( $10.8 \times 6.55$ ) per head, or 15.1 Calories per 100 kgm. live weight.

This value is about two and one-fourth times as great as that obtained with cow No. 874 (26.3 Calories per head, the cow weighing 400 kgm.).<sup>9</sup> The discrepancy between the present and the earlier determination is apparently due chiefly to the fact that in the previous study in the computation of the difference in heat production of standing and lying the total heat production was apportioned between the aggregates of intervals of standing and of lying in proportion to the CO<sub>2</sub> observed for these intervals. This assumes, in effect, that there was no instrumental lag affecting the CO<sub>2</sub> of the intervals of standing and lying involved in the computation, and that the CO<sub>2</sub> as observed correctly represented the heat production of these intervals. In the light of the present understanding it is now clear that in the earlier study an extensive instrumental lag, a "carry-over" from the previous interval, whatever the position of the animal, affected the validity of the observed CO<sub>2</sub>, as related to the intervals of standing and lying—the effect being, therefore, very materially to reduce the apparent difference in the CO<sub>2</sub> and the heat of standing and lying.

This study will be continued as a feature of the general program of calorimetric investigation of this institute.

#### SUMMARY

The determination of the energy expenditure of cattle in the standing compared with the lying position, as a basis for the computation of the heat production to a standard day as to standing and lying, has been reconsidered in the light of new evidence of the extent of the experimental lag, affecting the measurements of CO<sub>2</sub> and heat in the use of the respiration calorimeter.

A steer weighing 468 kgm. produced 10.8 liters more CO<sub>2</sub> per hour (2.31 liters per 100 kgm. live weight) during standing than during lying. On the basis of a determined heat-CO<sub>2</sub> ratio of 6.55, the increase of heat production for standing as compared with lying was 70.7 Calories per head, or 15.1 Calories per 100 kgm. live weight, per hour.

<sup>9</sup> FRIES, J. A., and KRISS, M. Op. cit.

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## TYLENCHUS PRATENSIS AND VARIOUS OTHER NEMAS ATTACKING PLANTS<sup>1</sup>

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### SYNONYMS AND ECONOMIC SIGNIFICANCE OF TYLENCHUS PRATENSIS

A large number of specimens from roots of various hosts were at hand for the study of *Tylenchus pratensis*.<sup>2</sup> This organism was described by De Man in 1884 as a rare species living free in the soil and at that time represented as having a definite posterior esophageal bulb, a statement which greatly influenced the further development of knowledge of this form (♂, tab. xxii, fig. 95).<sup>3</sup>

In 1917 Cobb (3) described *Tylenchus penetrans* as a root parasite of potatoes, violets, cotton, and the camphor tree, and in 1919 (4) he emphasized the close relationship of *T. pratensis*, *T. penetrans*, and *T. musicola*. In a later note in his files he came to the conclusion that *T. pratensis* and *T. penetrans* were identical. The material which led him to this view was lily-of-the-valley roots infested with *T. penetrans* from Wageningen, the Netherlands, which came, therefore, from the same country from which De Man had obtained his original material.

In 1924 *Aphelenchus neglectus* was described by Rensch (13) from various host plants in Germany. This species in the conception of the writer is identical with the material under consideration, which he considers beyond doubt to be *Tylenchus penetrans* of Cobb = *T. pratensis* of De Man.

In summarizing previous and present observations, *Tylenchus pratensis* must be regarded as an important widespread plant-parasitic nema of a pronouncedly destructive nature. It seems, therefore, that a clear definition of this species is of much importance.

Of all the descriptions given, that of Cobb is undoubtedly the most complete and accurate, and the specimens used in this study could easily be identified as his form. What apparently led him to create a new species under the name of *Tylenchus penetrans* was the very definite posterior esophageal bulb which De Man, one of the keenest of observers, draws in his figure of *T. pratensis*, together with the classification of the latter as a free-living form. However, Cobb, as well as the writer, has seen specimens of *T. penetrans* which

<sup>1</sup> Received for publication Aug. 15, 1927; issued January, 1928.

<sup>2</sup> For a part of the material dealt with in this paper the writer is indebted to E. G. Arzberger, of the Office of Nematology, who kindly called attention to several forms of nemas found during examinations of roots of a number of crop plants for their endophytic fungi. The grape roots infested with *Tylenchus musicola* were sent in by C. E. Scott, State of California Department of Agriculture, Sacramento, Calif. The lily-of-the-valley pips mentioned in connection with *Tylenchus pratensis*, the freesias infested with the root-knot nematode, as well as the cattleya plant mentioned later, were brought by Freeman Weiss, of the Office of Vegetable and Forage Diseases.

<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 981.

might account for De Man's drawings. In such specimens the glands and the tissue just in front of the intestine form a complex which could be conceived of as a posterior bulb. Although *T. penetrans* of Cobb, *Aphelenchus neglectus* of Rensch, and the writer's forms agree with *T. pratensis* of De Man in all other essential characters, they show this posterior esophageal bulb to be very indistinct. It can therefore be understood that Rensch, who had no males to examine and was looking for a well-developed posterior bulb, came to the conclusion that his specimen did not belong to *Tylenchus* but to *Aphelenchus*. However, if he had had more experience with free-living nemas, he would have noticed that the whole appearance and character of his specimens were more like those of *Tylenchus* than of *Aphelenchus*. There is, for example, the outlet of the salivary glands emptying dorsad into the esophageal tube just behind the spear—a feature not yet observed in any *Aphelenchus*. The figure Rensch gives is very rough and incomplete; the anal opening is not shown, nor the nerve ring, excretory pore, renette, or salivary glands. But all the facts Rensch gives about the size, morphology, and ecology of his animals agree so well with the writer's specimens that he considers them undoubtedly the same species. The writer, too, had not seen males until he had some fresh lily-of-the-valley pips, in which of 101 infesting specimens examined, 50 per cent were larval, 36 per cent females, and 14 per cent males, the sex number being 38.8. De Man mentions having had only one male; in Cobb's material, too, the females far outnumbered the males. This leads to the conclusion that they are either rare or have such different life habits from the females that they more easily escape observation. From a closer examination of conditions in lily-of-the-valley roots, it seems that the adult males more often stay on the surface of the roots or in the surface layers of the tissue. This points to the possibility that copulation takes place outside the root tissue.

Perhaps Rensch was unable to locate males, in spite of apparently plentiful material and after examining the surrounding soil, because he was looking for an *Aphelenchus* male to go with his supposed *Aphelenchus* females.

The fact that De Man's specimens were found living free can hardly be made a point against the identity of his and the writer's form. He had only three specimens and considered the form extremely rare; this corroborates the writer's observations of soil about the roots of *Sisyrinchium*, from which he was able to obtain only a few specimens, whereas within the roots they were fairly abundant.

A further point in favor of considering *Tylenchus penetrans* identical with *T. pratensis* is a note in Cobb's files referring to a cablegram from the Netherlands stating that *T. pratensis* was common there in roots of lily of the valley at the time Cobb examined roots of the same plant from Wageningen submitted by the Federal Horticultural Board. These lily-of-the-valley roots were infested with what he considered *T. penetrans*. The writer has observed the form in large numbers of lily-of-the-valley roots imported from Germany, where Rensch considered this *Aphelenchus neglectus* such a common pest. (Fig. 1.)

The situation to-day, therefore, is such that *Tylenchus pratensis* De Man 1884 must be considered as synonymous with *T. penetrans* Cobb 1917 and *Aphelenchus neglectus* Rensch 1924. The morphology of the form has best been worked out by Cobb. The writer has little to add to his description.

A study of living specimens revealed in an interesting way the ever-changing aspect which the annulation of the cuticle presents—dense with the contracted body and wide with the expanded. As Cobb remarks, the annulation continues even to the head end, which is set off in a buttonlike manner. (Fig. 2, A.) As many as three annules could be counted on the lip region. The cuticularized skeleton of this region is entirely interior and represents a very complicated framework. (Fig. 2, E.) The periphery of each lip is supported by an arcuate apophysis. This connects with a framework that surrounds the vestibulum and forms both a base to the lip region and a tubular structure backward, apparently some sort of guiding ring for the spear. Observing living specimens, it is noticed that the lip region is continually used as a sucking apparatus and in this case fixed on either the slide or the cover glass. Undoubtedly the whole framework is a means to develop these sucking qualities.

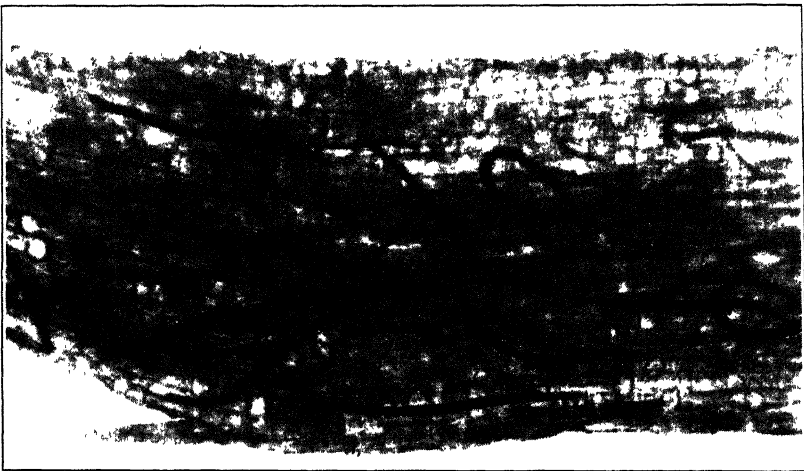


FIG. 1.—Portion of a lily-of-the-valley root infested with *Tylenchus pratensis* De Man.  $\times 60$

Micoletzky proposed in 1922 (10, p. 542) to place all the Tylenchi which have a cuticularized framework in the lip region in a separate subgenus, *Chitinotylenchus*. In 1925 (11, p. 255), however, he declared his subgenus *Chitinotylenchus* to be synonymous with Cobb's genus *Tylenchorhynchus* (2, p. 438). The latter genus, however, in its type species has two ovaries—a character which is not shared by all the members of *Chitinotylenchus*. As a generic or subgeneric character, this is considered more valuable than the cuticularized framework in the lip region. Therefore, it is perhaps better to regard *Tylenchorhynchus* distinct from *Chitinotylenchus*.

The writer was able to locate the amphids, although they are extremely small (Fig. 2, B.) They open on the exterior half of the lateral lip with an oval aperture. From here inward there leads a fine tubular canal, with thickened walls, ending just behind the lip region. Then follows a thin-walled section which resembles an extremely slender, elongated bottle. It could be followed back to a

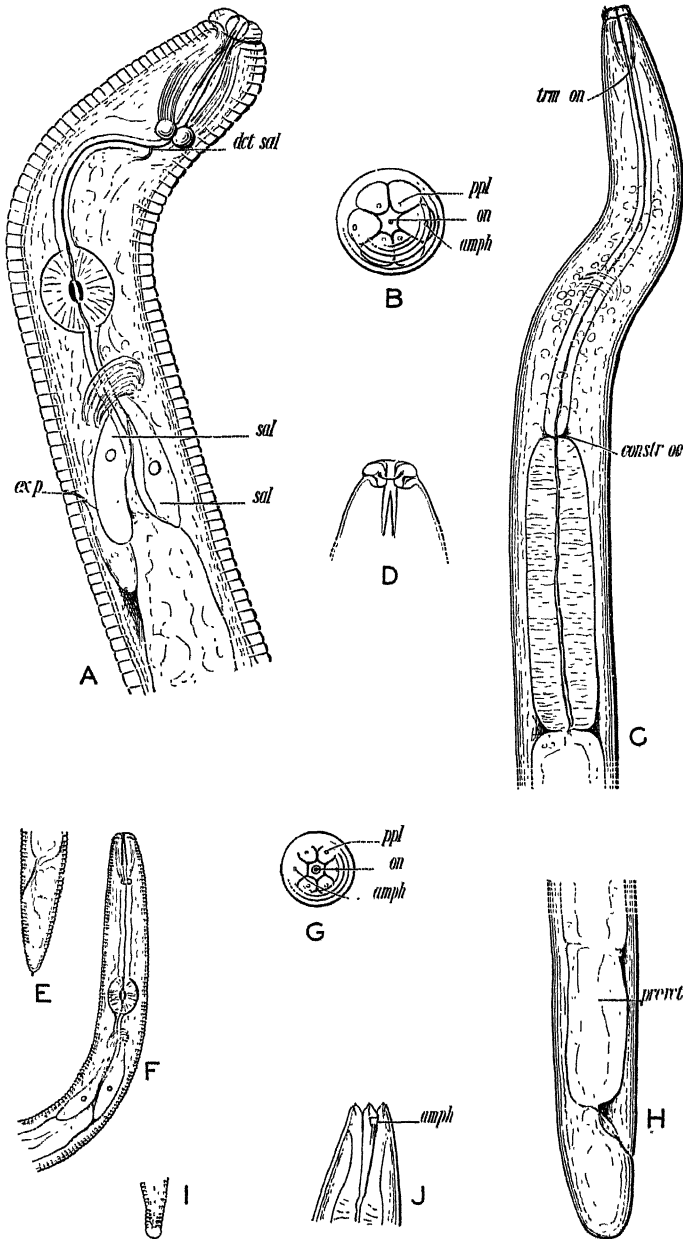


FIG. 2.—A, Anterior end of *Tylenchus pratensis* De Man; *det sal*, outlet of the salivary glands; *ex p*, excretory pore; *sal*, salivary glands.  $\times$  about 1,400.  
 B, Front view of head of same species; *amph*, amphid; *on*, spear; *ppl*, head papillae.  $\times$  about 2,100.  
 D, Molt of the head end of same species.  $\times$  about 1,400.  
 F, Anterior end of *Tylenchus musicola* Cobb.  $\times$  about 500.  
 G, Front view of head of same species; *amph*, amphid; *on*, spear; *ppl*, head papillae.  $\times$  about 2,100.  
 E, Tail end of a larval specimen of same species.  $\times$  about 500.  
 I, Tail end of an adult female of same species.  $\times$  about 500.  
 J, Head end of *Cephalobus oxyuroides* De Man; *amph*, amphid.  $\times$  about 1,400.  
 C, Anterior end of *Axonchium parvum* n. sp.; *constr oe*, constriction of the esophagus (typical for this genus); *trm on*, end of spear.  $\times$  about 700.  
 H, Tail end of same species; *prerect*, prerectum.  $\times$  about 700.

short distance behind the base of the spear. A number of fibers (terminals) were seen inside. The whole organ is extremely fine and was best seen in specimens in exitu.

Head papillae were not seen. Even in front views it was not possible to ascertain their presence.

Figure 2, D, shows the molted head cuticle, which is of some interest because all the framework of the lip region is contained in this molt. As Cobb has described it from other *Tylenchi*, the spear is molted only in its anterior conical part, which is distinctly set off from the cylindrical posterior part. Cobb's conception of the difference in origin of the anterior part of the spear and the posterior part seems therefore well founded.

It was noticed that the lateral wings, too, were annulated, but that here one annule was as wide as two of the rest of the cuticle; the space between the wings also shows longitudinal striae, at least one on each side. In one specimen a much larger number was seen in the vulvar region.

As has been remarked, De Man found his few specimens free in the soil. Cobb (3) in his paper on *Tylenchus penetrans* gives four important hosts, and in his unpublished files two others, the lily of the valley and the fig tree. Rensch mentions a number of hosts from Germany and notes their pathological features. They consist of restricted growth and yellowing of the leaves—features which most often are attributed to other causes than nema infestations. This may be one reason why *T. pratensis* as a plant parasite has hitherto been given so little attention by plant pathologists, although it appears to be a very widespread and serious nemie pest. The host list already includes the following plants:

Cobb's observations:

- Potato tubers (Michigan, Tennessee).
- Violet roots (New York).
- Cotton roots (South Carolina, Georgia).
- Camphor-tree roots (Florida).
- Lily-of-the-valley roots (Wageningen, the Netherlands). Unpublished.
- Ficus carica*, fig-tree roots (California).

Rensch's observations:

- Winter rye (Germany).
- Wheat (Germany).
- Winter barley (Germany).
- Weingartneria canescens* (Germany).
- Papaver plants (Germany).
- Wild oats (Germany).
- Atriplex* plants (Germany).
- Sugar beets (Germany).
- Rape (Germany).

Writer's observations:

- Wheat (Arlington Experiment Farm, Rosslyn, Va., and Ohio Agricultural Experiment Station).
- Rye (Arlington Experiment Farm, Rosslyn, Va.).
- Corn (*Zea mays*) (near Chain Bridge, Va.).
- Sugar cane (greenhouse, Arlington Experiment Farm, Rosslyn, Va.).
- Cotton (South Carolina).
- Poplar hybrids (propagation plot of New York Botanical Garden).
- Sisyrinchium angustifolium* (Woods Hole, Mass.).
- Lily of the valley (*Convallaria majalis*; Germany).
- Cattleya sp. (greenhouse near New York City).

The measurements given by the various authors are as follows:

De Man:

$$\frac{2.7}{2.4} \quad \frac{?}{?} \quad \frac{22.6}{3.6} \quad \frac{-79-}{3.6} \quad \frac{96.4}{2} \quad 0.6 \text{ mm.}$$

$$\frac{3.3}{?} \quad \frac{?}{?} \quad \frac{20}{?} \quad \frac{M}{3.6} \quad \frac{95}{2.2} \quad 0.45 \text{ mm.}$$

Cobb:

$$\frac{2.8}{3.1} \quad \frac{12}{4.2} \quad \frac{18}{4.5} \quad \frac{5078^5}{4.6} \quad \frac{95}{2.6} \quad 0.7 \text{ mm.}$$

$$\frac{3.2}{4} \quad \frac{11}{4.5} \quad \frac{16}{4.6} \quad \frac{M}{4.3} \quad \frac{94.5}{3.6} \quad 0.5 \text{ mm.}$$

Eggs.—Length, 0.078 mm.; width, 0.025 mm.

Rensch:

$$\frac{3.9}{3.9} \quad \frac{?}{?} \quad \frac{13}{?} \quad \frac{-75-}{4.9} \quad \frac{?}{?} \quad 0.467 \text{ mm.}$$

Eggs.—Length, 0.063 to 0.068 mm.; width, 0.021 to 0.025 mm.

Measurements from the writer's specimens:

Average of three females—

$$\frac{2.9}{2.5} \quad \frac{12}{3.4} \quad \frac{17}{3.5} \quad \frac{80}{3.7} \quad \frac{95.6}{2.5} \quad 0.58 \text{ mm.}$$

Eggs (average of 5).—Length, 0.067 mm.; width, 0.025 mm.

Average of three males—

$$\frac{3.2}{2.2} \quad \frac{15}{3} \quad \frac{20}{3.2} \quad \frac{M}{3.2} \quad \frac{95.8}{2.4} \quad 0.5 \text{ mm.}$$

As pathological symptoms resulting from infestation by the present species, Cobb described peculiar pustules on potato tubers and brownish spots on the roots of violets. Such spots have been seen by the writer on roots of *Sisyrinchium*. In the lily of the valley, however, the spots, or sometimes whole areas, were reddish. With the exception of potato tubers, the nemas have been observed only in the cortex of roots, where they move in the parenchymal tissue and apparently feed on the cell contents. They force their way through the cell walls; often, especially in cross sections, one comes across a specimen which has its body "strangled" by a cell wall which did not give way; but this apparently does not matter to the nema.

Very often one sees a large number of specimens quite close together, as shown in Figures 3, 4, and 5. Eggs, larvae, and adult females are seen together; as many as five eggs have been seen deposited tandem, separated by only a few plant cells. The eggs are large compared with the adults, but rather thin shelled. They develop inside the roots; the larvae hatch out blunt tailed and devoid of any signs of a spinneret. In agreement with the other observers, the writer has seen only a single egg at a time in the uterus.

Cobb has already emphasized the economic significance of the present species. Rensch considers the present species very inju-

rious, especially to young plants and seedlings. He found the roots of seedlings only four weeks after they had been sown completely shot through with the nemas. But it is only necessary to look at Figures 1, 3, 4, and 5, which represent typical cases of the way these nemas occur in the finer roots, to realize that such a plant can not grow properly. The writer is convinced that the future will prove that in many instances troubles caused by the present form have wrongly been laid to some other cause.

Specimens of *Tylenchus pratensis* undoubtedly move slowly but continually through the root tissues. The path prepared by the larvae aids in the spread of other root diseases through the root tissues, especially fungi. Cases have been observed by the writer where numerous chytrids could be seen following what appeared to be the trail of *T. pratensis*.

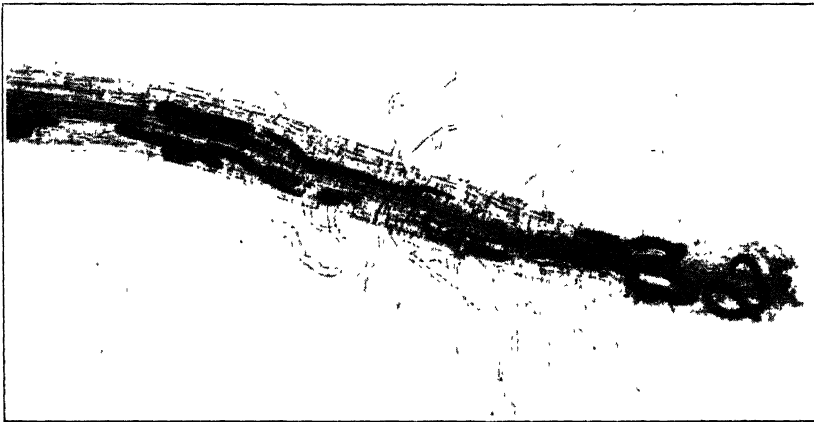


FIG. 3.—Root of wheat (Kanred) infested with *Tylenchus pratensis*.  $\times$  about 55

#### HOPLOLAIMUS CORONATUS, A PARASITE IN ROOTS OF SUGAR CANE, CORN, RED CLOVER, AND ALFALFA

*Hoplolaimus coronatus* was only recently (1923) described by Cobb (5) from a few specimens found around a "nest of Mermis" near Falls Church, Va. The comparatively late discovery of this large and outstanding nema is probably due to its parasitic life, which keeps the specimens inside the roots of their host plants during most of their life period. The writer has frequently seen specimens leaving roots or just on the point of entering them. (Fig. 6.) This observation rather points to a vagrant mode of life. It is not known whether this species copulates inside or outside the roots, but its considerable size would indicate that copulation in the soil is more probable.

*Hoplolaimus coronatus* Cobb was first observed by the writer in roots of corn (*Zea mays*) from Virginia and was found to spread all through the fine roots; later it was noticed to be very common and abundant in roots of red clover and alfalfa from near the campus of the University of Maryland, College Park, Md.<sup>4</sup> In 1924 it was also found in roots of sugar cane grown in greenhouses at the Arlington

<sup>4</sup> For these samples the writer is indebted to H. S. McConnell, Division of Entomology, University of Maryland.

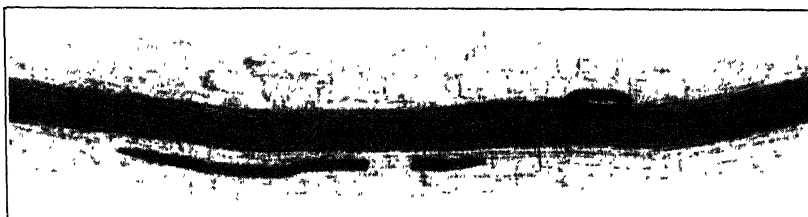


FIG. 4.—Root of rye infested with *Tylenchus pratensis*.  $\times$  about 4 or 5

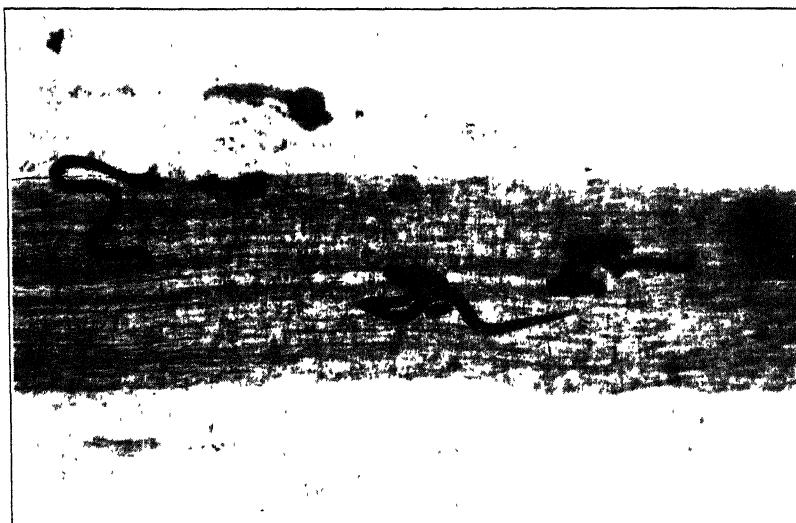


FIG. 5.—Root of a cotton plant infested with *Tylenchus pratensis*.  $\times$  about 55

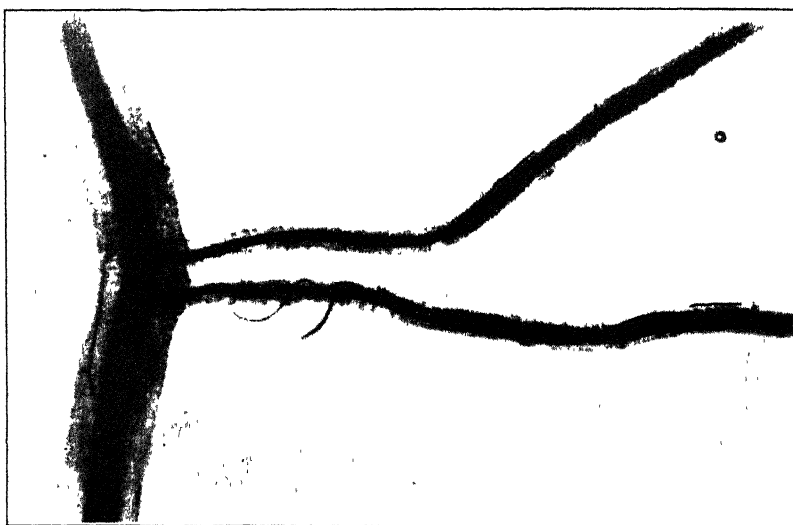


FIG. 6.—Corn roots with *Hoplolaimus coronatus* Cobb. Two specimens are entering the roots.  
 $\times$  about 8

Experiment Farm, Rosslyn, Va. In a recent paper F. Muir mentions two *Hoplolaimi* from sugar-cane roots from Hawaii but does not name the species of *Hoplolaimus*. Within the roots, these nemas are always located in the parenchyma of the cortex, and the writer has never seen them elsewhere. Although a few specimens may occasionally be found together, this form never occurs in "nests," like *Tylenchus pratensis*.

*Hoplolaimus coronatus* Cobb is probably widespread, attacking a much larger number of plants than the few mentioned above. Its significance may not be limited to the destruction of the root tissues and interference with the physiological function of the roots. On account of its considerable size it may be an agent which prepares an entrance for other diseases and thus aids in spreading them.

#### TYLENCHUS MUSICOLA LIVING IN ROOTS OF GRAPE

It was reported by Cobb in 1918 that *Tylenchus musicola* Cobb from roots of the Bluggoe banana, Grenada, Barbados, West Indies, caused a serious disease. The present findings from roots of grapes (*Vitis vinifera*) from Loomis, Calif., are the second ones, thus showing that this nema is not confined to the banana and the Tropics.

The nemas were found all through the parenchymal tissue, close to the central cylinder; larvae and females were observed, but no males were found. The grape roots had swollen tips and were therefore examined for root knot (*Caconema radicolola*), which led to the finding of the infestation by the present form. Unfortunately, the roots as received were quite dry; hence the material was not of the very best. Cross sections proved that the cell walls were greatly damaged and apparently broken to a considerable extent by the moving nemas. Seemingly the cells are emptied of the plasmatic content.

The characters given by Cobb apply very well to the specimens received. (Fig. 2, D.) The writer is convinced that not only "phasmids" (lateral papillalike organs in the middle of the tail end in the male and female) but also "deirids" (lateral papillae in the cervical region) are present; the latter were observed in the latitude of the anterior esophageal bulb. Regarding the spear, Cobb writes: "In the preserved specimens the anterior portion of the spear is distinctly visible, but the bulbs and hilt are rather ghostly, though well developed." This characterizes in the most accurate way the conditions observed in the writer's specimens. The posterior esophageal bulb is very indistinct. The salivary glands were seen, but not their outlet behind the base of the spear. However, an ampullalike swelling of a glandular nature was observed inside the esophageal tissue. Cobb has already emphasized the variability of the shape of the tail end. In the writer's material the tail was usually bluntly rounded, as shown in Figure 2, I. In the larval specimens a small mucro was often seen (fig. 2, E); sometimes this mucro was shifted to the ventral side of the then rather blunt end.

In studying the front view of the head (fig. 2, G), four submedial but rather faint papillae were seen. The amphids are also very inconspicuous and somewhat closer to the periphery of the head. The measurements were found to be as follows:

Female 1:	2.6	12	19	79	95.5	0.64 mm.
	1.9	2.9	3.2	3.2	2.3	

Female 2:	$\frac{3}{2}$	$\frac{14}{3}$	$\frac{21}{3.4}$	$\frac{79}{3}$	$\frac{93.9}{2.3}$	0.54 mm.
Female 3:	$\frac{3.1}{2.3}$	$\frac{14}{3.1}$	$\frac{23}{3.5}$	$\frac{78}{3.5}$	$\frac{93.8}{2.7}$	0.52 mm.

#### RELATIONSHIP OF SOME SO-CALLED SOIL NEMAS TO CORN SEEDLINGS

For the study of the relationship of some chytrids to corn roots, the following experiment was arranged:<sup>5</sup> A handful of soil was placed in the bottom of a cylindrical glass jar about 75 cm. in height. The soil was known to contain chytrids, but no special observations were made concerning its nemic fauna, although it was known that *Hoplolaimus coronatus* was present at the place where the soil was collected. After the soil was carefully placed in the bottom of the jar, the latter was cautiously filled with running water and a corn seed placed on top of the water column now about 66 cm. high. The seed itself had been treated with bichloride of mercury. After four weeks the seedling was removed and the roots subjected to a very careful examination. The search revealed a number of nematodes within the roots. From a piece of root about 2½ cm. long 58 specimens were isolated. The forms observed belonged to six different species: 1, *Dorylaimus brigdammensis* De Man; 1, *Axonchium parvum* n. sp.; 2, *Aphelenchus parietinus* Bastian; 18, *Cephalobus oxyuroides* De Man; 1, *Plectus granulatus* Bastian; 35, *Plectus longicaudatus* Bütschli.

These findings are of some significance because they throw a more definite light upon the relationship of the forms mentioned to plants, especially to corn. In phytopathological circles usually very little consideration is given to the so-called soil nemas. All the above-mentioned forms, however, belong to this group and have a much closer relationship to plant life than was once thought. The nemas were apparently able to locate the growing seedling, which at the beginning was at least 60 cm. distant. The attraction which the seedling exerted was probably based on chemical secretions which diffused through the water and served as a directing agent. To cross the distance the nemas had either to move in an almost perpendicular path through the water or to use the wall of the glass jar as a climbing support. But in either case what appears to be a developed sense of "smell" is rather outstanding. This point calls for even more consideration because the forms involved were not thought to have plant-attacking habits. It must be admitted that the soil used in this experiment was taken from a cornfield around the corn roots. The nemas had therefore probably been familiar with corn as a food plant and so may have responded more accurately to it than they would have responded to any other plant.

Of all the species present, *Plectus longicaudatus* Bütschli was most numerous; 12 adult females and 23 larvae of various sizes were observed; 7 females had eggs. Contrary to observations of earlier investigators (7, 10), most of the specimens had distinct lips. This would bring them closer to *P. rhizophilus* De Man, or, as Micoletzky conceived it, to *P. cirratus* Bastian var. *rhizophilus* De Man; but the size, the shape of the tail, the structural conditions of the cardiac

<sup>5</sup> By E. G. Arzberger, Office of Nematology.

bulb, the mouth cavity, and the amphids rather speak for systematic position near *P. longicaudatus*. The writer can support the description of the amphids which De Man gave in 1921 (8); their external parts have a spiral form with only one turn rather than a circular form. From this part a duct can be followed leading backward as far as a short distance proximad to the base of the mouth cavity; the duct always starts at the open edge of the spiral, i. e., it is always dorsad. Another observation concerning the morphology of *P. longicaudatus* is the presence of a deirid, i. e., a fine lateral papilla on each side a short distance behind the nerve ring. Such papillae have not yet been mentioned as occurring in any of the members of the genus *Plectus*.

The number of specimens of *Plectus longicaudatus* present in the roots of corn seedlings proves beyond any doubt its ability to feed entirely on corn roots and to live and propagate within the root tissue. At least some of the younger larvae seem to have developed from eggs which the adult forms had deposited within the roots. *P. longicaudatus* is syngonic, and therefore propagation is not dependent upon the presence of males. This may account for the fact that *P. longicaudatus* was more numerous in the present case than *Cephalobus oxyuroides*. A careful study of the intestinal content of the specimens was made, but only finely granulated materials were seen. It is therefore concluded that *P. longicaudatus* fed directly on the cell contents of the root parenchyma in which it was found. Although the water roots of the present seedling represented especially soft tissue, this alone would not account for the presence of all the nemas in it. There is certainly a definite food relationship between the nemas studied and the corn plant. The measurements of two adult specimens of *P. longicaudatus* were as follows:

0.54	15	27	'51'	85	0.38 mm.
1.3	3.2	4.3	5	2.2	
0.66	13	25	50	86	0.43 mm.
1.2	3.3	4.1	4.7	1.9	

These measurements agree well with those given by earlier observers—a fact which indicates normal living conditions in the present case.

*Cephalobus oxyuroides* De Man was next in number to *Plectus longicaudatus*. It is a species which had been observed by the writer very frequently in various tissues of numerous plants. This form also seems able to live upon plants entirely. The corn roots studied contained 7 adults (4 males and 3 females) as well as 11 larval forms, and it is thought that copulation and the deposition of eggs occurred within the root parenchyma.

The head end of the present species, shown in Figure 2, H, J, may aid in completing our knowledge of the head sense organs. As can be seen, the six lips have two fine papillae each; the amphids are situated on the dorsal part of the lateral lip, close behind the lateral papillae. This species too has phasmids (lateral papillalike organs in the middle of the tail in males as well as females) and deirids.

The writer has already called attention to the economic significance of various other members of the genus *Cephalobus* in other papers. The present observations afford additional evidence. Many Ceph-

lobi, the present species included, may be omniphagous, at least to a certain degree. Although this character makes less significant the extent of direct damage done by this species of nemas, on the other hand it causes them to serve as important carriers for diseases (bacterial, virus(?), and fungous).

*Dorylaimus brigdammensis* De Man was found, but only a single specimen. It had not yet been recorded from America, but was known to live in the soils of the Netherlands, Switzerland, Denmark, and Russia. Its food and feeding habits were not known. The present observation suggests a parasitic relationship to plants. The specimen had an egg in its uterus. The measurements were as follows:

$$\frac{0.14 \quad 7.4 \quad 22 \quad \text{---}48\text{---} \quad 83}{0.69 \quad 1.8 \quad 2.2 \quad 2.2 \quad 1.5} \quad 1.5 \text{ mm.}$$

*Axonchium parvum* n. sp. is the smallest of all known members of this genus. Although some *Axonchia* had been found around the roots of plants, none had been observed in plant tissue.<sup>6</sup> The present specimen was an adult female, but had no egg in the uterus, although one was ready to enter it. Doubtless this form can live entirely parasitically.

The distinguishing characters of the new species are as follows:

$$\text{Measurements.---} \frac{0.64 \quad 8.7 \quad 27 \quad 51\text{---}}{1.1 \quad 2.9 \quad 3.5 \quad 3.7} \frac{97.4}{2.2} \quad 0.64 \text{ mm.}$$

*Diagnosis.*—*Axonchium* of small size (0.64 mm.); the enlarged posterior part of the esophagus less than one-half its length; tail end bluntly rounded, ovary posterior, with the end reflexed and reaching two-thirds back to the vulva; male unknown.

Only a few species of this well-defined genus<sup>7</sup> are described. The present one is much smaller than all the others and especially well characterized by the short posterior part of the esophagus. It is very well set off by the deep constriction so typical of the genus. The writer was unable to determine definitely the number of head papillae, but estimates that there are two circle of six each, the lips being only faintly visible. The spear is about three times as long as the head is wide and, at least in the present specimen, rather fine. (Fig. 2, C and H.)

*Plectus granulatus* Bastian was represented in the corn roots by a single adult female with the following measurements:

$$\frac{2.2 \quad ? \quad 21 \quad 14 \quad 52 \quad 15 \quad 92.3}{1.8 \quad ? \quad 3.3 \quad 3.6 \quad 2.2} \quad 1.02 \text{ mm.}$$

This form has already been claimed by several observers as feeding, at least partly, in plants. It has been mentioned from seedlings of cereals, and Micoletzky (10) found chlorophyll as intestinal content.

*Aphelenchus parietinus* Bastian was represented by an adult female with one egg and a very young larval specimen. The measurements of the female were as follows:

$$\frac{1.8 \quad 4.9 \quad 12 \quad 38 \quad 67 \quad 91.3}{1.8 \quad 2.3 \quad 2.7 \quad 2.9 \quad 1.5} \quad 0.42 \text{ mm.}$$

<sup>6</sup> Since this was written F. Muir states that *Axonchium* was living in sugar-cane roots in Hawaii (12), but he does not mention the species.

<sup>7</sup> It is a great mistake to consider this well-characterized genus as synonymous with *Dorylaimus*, as do Baylis and Daubney in a recently published book (1).

Following Micoletzky's conception of this species, the present form would come under the variety *tubifer*, forma *parvus*, subforma *informis*. *A. parietinus* has been recorded from various hosts by several investigators.

#### UNUSUAL REACTIONS OF FREESIAS TOWARD THE ROOT-KNOT NEMATODE (*CACONEMA RADICICOLA*)

A conception commonly found among plant pathologists is that nemec diseases of plants can be recognized with much accuracy by certain pathological symptoms exhibited by the host plants. This is often the case, but appearances are also often misleading.

Plants with nemec infestation might sometimes (perhaps under certain climatic or growth conditions) show no symptoms, and in other cases symptoms regarded as typical for nemec diseases might exist and no nemas be found. Nematologists therefore are rather skeptical about such symptoms and consider an examination with the microscope the only safe way to determine the presence of nemas. Besides, as already shown in the present paper, a number of injurious plant-parasitic nemec species never produce special symptoms, i. e., symptoms which could not be attributed also to some other cause.

Even such a nemec disease as root knot, seemingly well characterized by its symptoms, is not always properly recognizable by them. Peonies, for example, have been observed in this laboratory in numerous instances to exhibit root swellings with all the typical features of root knot, and yet *Caconema radiculicola* could not be located. On the other hand, roots of plants, e. g., cyclamen, have also been observed which, although they showed no swellings harbored the root-knot nema and were severely affected. It is evident that various plants do not react in the same way toward the root-knot nema. Even early observers noted that some heavily infested plants seemed to suffer little or not at all, whereas other species with comparatively few knots were in a dying condition. Cyclamen is in this respect an outstanding example. It was observed that a few specimens of *Caconema radiculicola* were able to kill half-grown plants. The roots did not develop the typical knots, but began to decay at the places where the parasite was located; in some such instances the nemas could be located only after search somewhat more tedious than usual, because the absence of swellings left the observer without the usual characteristic guide.

Recently the writer came into possession of some freesia bulbs sent in for examination by a New Jersey nursery because of their failure to bloom after forcing. The bulbs were infested with *Caconema radiculicola*. This plant may be added therefore to about 660 other hosts already known. Most interesting, however, were the symptoms of this infestation. Contrary to the usual development where the whole root swells at the location of the parasite, such swellings were absent. In the fully developed stage brownish-yellow spherical bodies are attached to the roots. (Fig. 7.) They stand out especially well on the finer rootlets. At first sight they resemble the brown cysts of the sugar-beet nematode (*Heterodera schachtii*) and could be mistaken for such by inexperienced observers. However, a close examination of the adult female in these globules as well as of the larvae proves at once that it is the typical root-knot nema.

A comparison of such nodules at different stages of development (figs. 7 to 9) leads to an understanding of these anomalous conditions. In plants with a normal reaction toward the nema and the young female *Caconema* develops within the parenchyma of the root cortex. As the female begins to grow and swell, it presses toward the surrounding tissue, which naturally slackens on the side of least resistance, that is, toward the surface. Here a more and more pronounced elevation is formed. The tissue of freesias, however, seems to be unable to counteract or neutralize this expansion by regenerative action and simply cracks and splits, the outer tissue assuming a brownish discoloration. Eventually it dies off and forms the above-mentioned yellow-brown crust around the female nema,

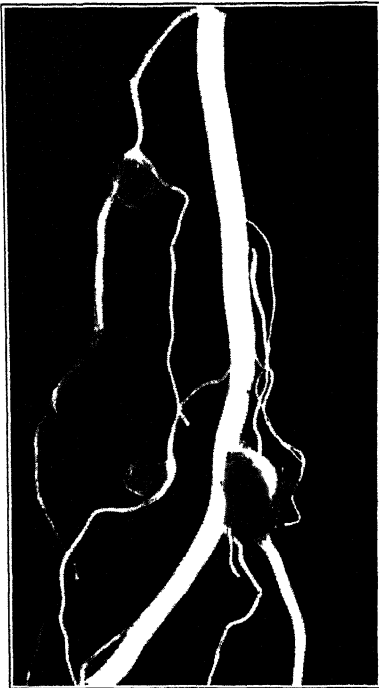


FIG. 7.—Roots of freesias with the females of *Caconema radiculicola* attached outside the roots  
 $\times 4\frac{1}{2}$

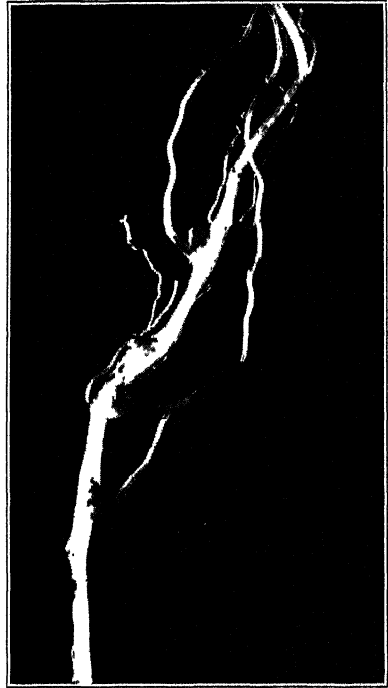


FIG. 8.—Roots of freesias with immature females of *Caconema radiculicola*.  $\times 4\frac{1}{2}$

which appears more or less outside the surface of the root and at least partially free. These conditions are understood to be a result of the lack of regenerative power in the root cortex tissue of freesias. Whether this is a general lack or a specific absence resulting from the influence of the nema, the writer is unable to judge. Freesia, however, evidently suffers much from a few *Caconema* parasites and belongs to that group of plants easily damaged by even a small infestation. It seems from the foregoing description that the symptoms are more nearly those of *Heterodera schachtii* than those regarded as typical for *Caconema radiculicola*, proving once more that too much stress should not be laid upon the macroscopic pathological symptoms in diagnosing nemic diseases.

REMARKABLE HABITAT OF *TYLENCHUS DIPSACI*

The stems, leaves, flowers, and seeds as well as certain subterranean stems and leaves (bulbs, tubers, and rhizomes) are the parts of plants usually chosen by *Tylenchus dipsaci* to live in. The recognition of bulbs, tubers, and rhizomes as types of stems and leaves proves much for the "botanical" instinct of this nema. So far as the writer knows, *T. dipsaci* has never heretofore been recorded from the roots of plants. The recent discovery of this nema in the roots of lilac

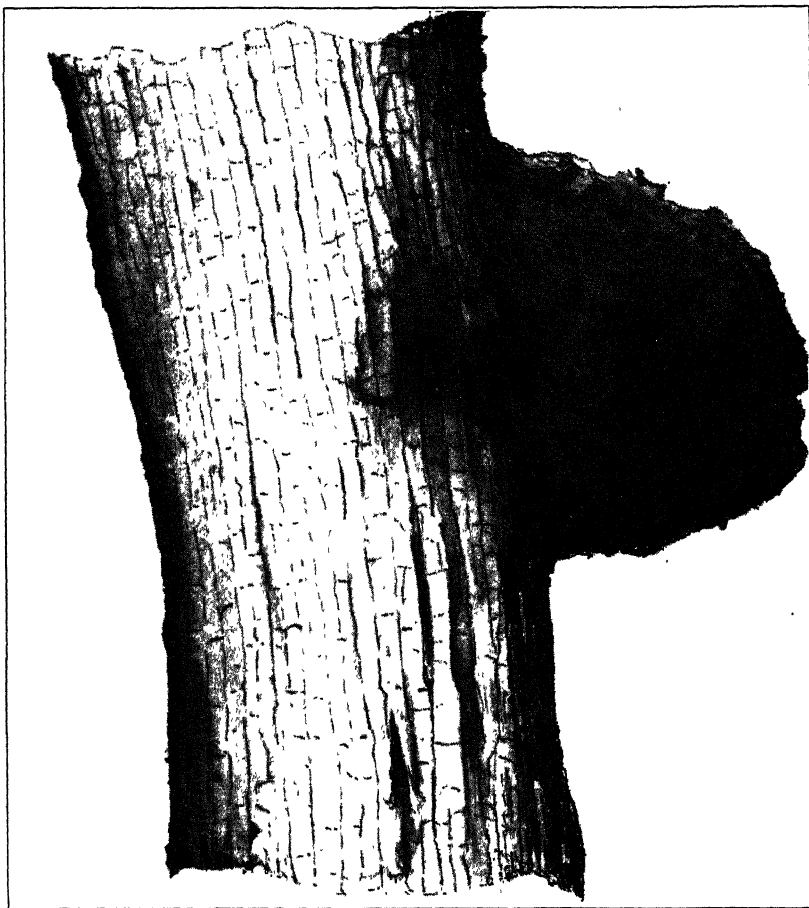


FIG. 9.—A single female of *Caconema radicicola* just breaking through the root surface of a freesia.  
X about 60

hybrids (*Syringa vulgaris*) therefore came as a surprise. These plants were imported from Nancy, France, and were submitted for examination because of warty outgrowths on the roots (crown galls). Nemas were also found, and these proved to be *T. dipsaci*. Although their number was not large, several were obtained, mostly larvae. At least a part of the specimens came from the above-mentioned warts.

The measurements of an adult male were as follows:

1.2	9.1	17	M	90.9	0.7 mm.
1.4	2.5	2.8	3.3	2	

## NEMAS ASSOCIATED WITH THE ROOTS OF AN ORCHID

A cattleya plant sent in for examination from a greenhouse near New York City afforded the opportunity to investigate, in a preliminary way, the nemic fauna associated with its roots. The cattleya, especially its root system and the soil in which it grows, undoubtedly represents highly specialized conditions for the life environment of a nemic fauna. It was therefore expected that a number of widely different forms, able to live under the most varied conditions, would be found. On the other hand, it was thought that perhaps some forms particularly adapted to the specialized conditions here existing might be observed. These expectations were realized. As may be seen from the list here given, most of the nemas isolated are forms able to live under most varied conditions. But there are also two forms which perhaps are peculiar to such orchids, namely, *Aphelenchus tenuicaudatus* De Man and *Diplogaster asymmetricus* n. sp. The former was described by De Man from the roots of orchids, namely, various species and hybrids of calanthes, and has never been found since, as far as the writer knows. *Diplogaster asymmetricus* is a new species and was the most numerous species present, a fact which seems to indicate that it is perhaps a form specific for orchids.

The nemas listed below all came from the surface or from the inside of the roots, only parts of which were examined. Their close connection with these roots is beyond doubt, although the exact nature of this relationship is not known. But that these relations might often be of indirect and complex character is to be concluded from the fact that hyphae of a mycelium were observed in the intestine of a *Plectus communis* found on these cattleya roots. This suggests that this nemic species is associated with the mycorrhiza of this orchid—a new viewpoint for the ecological significance of the nemic fauna of soils.

The nemas isolated from the roots of the cattleya plant included 100 specimens belonging to 15 different species and to 10 different genera, as follows: 14, *Rhabditis monhystera* Bütschli; 2, *Plectus longicaudatus* Bütschli; 1, *Plectus communis* Bütschli; 21, *Cephalobus oxyuroides* De Man; 5, *Cephalobus longicaudatus* Bütschli; 6, *Acrobeles* (*Acrobeloides*) *minor* Thorne;<sup>8</sup> 2, *Acrobeles tricornis* Thorne;<sup>8</sup> 26, *Diplogaster asymmetricus* n. sp.; 1, *Prismatolaimus intermedius* De Man; 1, *Bastiania longicaudata* De Man; 3, *Monhystera vulgaris* De Man; 3, *Tylenchus pratensis* De Man; 1, *Tylenchus intermedius* De Man; 13, *Aphelenchus tenuicaudatus* De Man; 1, *Aphelenchus* sp.

Thus the nemic component of the root biocoenosis of cattleya proves itself to be rich in numbers as well as in forms.

## RHABDITIS MONHYSTERA

A number of larval and female specimens of *Rhabditis monhystera* Bütschli were seen. De Man (9) has recently called attention to the fact that in the past at least three different species were wrongly placed under this name. The writer's specimens, however, surely belong to Bütschli's form.

<sup>8</sup> For the identification of these species the writer is indebted to Gerald Thorne, of the Office of Nematology.

## PLECTUS COMMUNIS

Only a single specimen of *Plectus communis* Bütschli was found—an adult female. Deiride are present; possibly also phasmids. The amphidial openings are not transverse oval openings as would be expected from De Man's drawings, but they have the shape of a defective spiral, open dorsoposteriorly. This form seems to live, at least partly, on fungus mycelium. A partly digested mycelial thread was distinctly seen in the intestine.

## CEPHALOBUS OXYUROIDES

Some specimens of the present material are considered to be *Cephalobus oxyuroides* De Man, although they are of very dwarfed size—such as not yet mentioned in literature. Of three males seen and measured, the smallest was only 0.38 mm. and the largest 0.41 mm.; yet, as may be seen from the following formulae, the size relations agree fairly well with what is known:

Male 1:	2.6	22	30	M	91.4	
	2.2	3.2	3.8	4.3	3.2	0.38 mm.
Male 2:	2.4	21	30	M	90.3	
	2.2	4.1	4.1	4.9	3.2	0.41 mm.
Female (fertilized, but without egg):	1.1	18	30	<sup>24</sup> 66 <sup>21</sup>	91	
	1.2	3.1	3.3	3.4	1.9	0.42 mm.

The three papillae on the male tail end just in front of the terminal point could always be detected easily; this was not true of the other papillae, which were very faint and sometimes rather doubtful.

## DIPLOGASTER ASYMMETRICUS

A considerable number of female, male, and larval specimens of the new species, *Diplogaster asymmetricus* n. sp., were seen. (Fig. 10, A, C, D, E.) Its name was chosen because of the asymmetrical position of the pharyngeal teeth.

## Measurements—

Female:	0.93	9.3	14	<sup>17</sup> 41 <sup>18</sup>	74	
	1.2	1.9	2.3	3.2	1.8	0.88 mm.
Male:	0.75	9	14	M	71	
	1.2	1.9	2	2.3	1.8	0.82 mm.

*Diagnosis.*—Diplogaster with a very elongated setaceous tail end (fig. 10, E) in both sexes; a mouth cavity wider than deep and about half as deep as the head is wide; only two pharyngeal teeth, one somewhat larger dorsally, slightly to the left and a submedial smaller one also to the left (fig. 10, C); middle esophageal bulb well developed (fig. 10, D); cardiac bulb indistinct, in fact only a gradual swelling of the posterior part of the esophagus; male with curved spicula and a somewhat flag-shaped gubernaculum, the staff of which points along the spicula to the anal opening, whereas the flag is directed caudad and has a rounded edge; a ventro-submedial setaceous papilla is in front of the inner end of the spicula, a second one in front of the anus, a third one back of the anus, and a group of three in about the middle of the conical, not filiform, part of the tail; there are also three lateral papillae, one in front of the anus, one close to the middle of the conical part of the tail and one about where the filiform tail end begins (fig. 10, E).

*Description and remarks.*—The tail end of this species is extremely fine, but breaks off apparently quite easily. It is not difficult to recognize the annulation

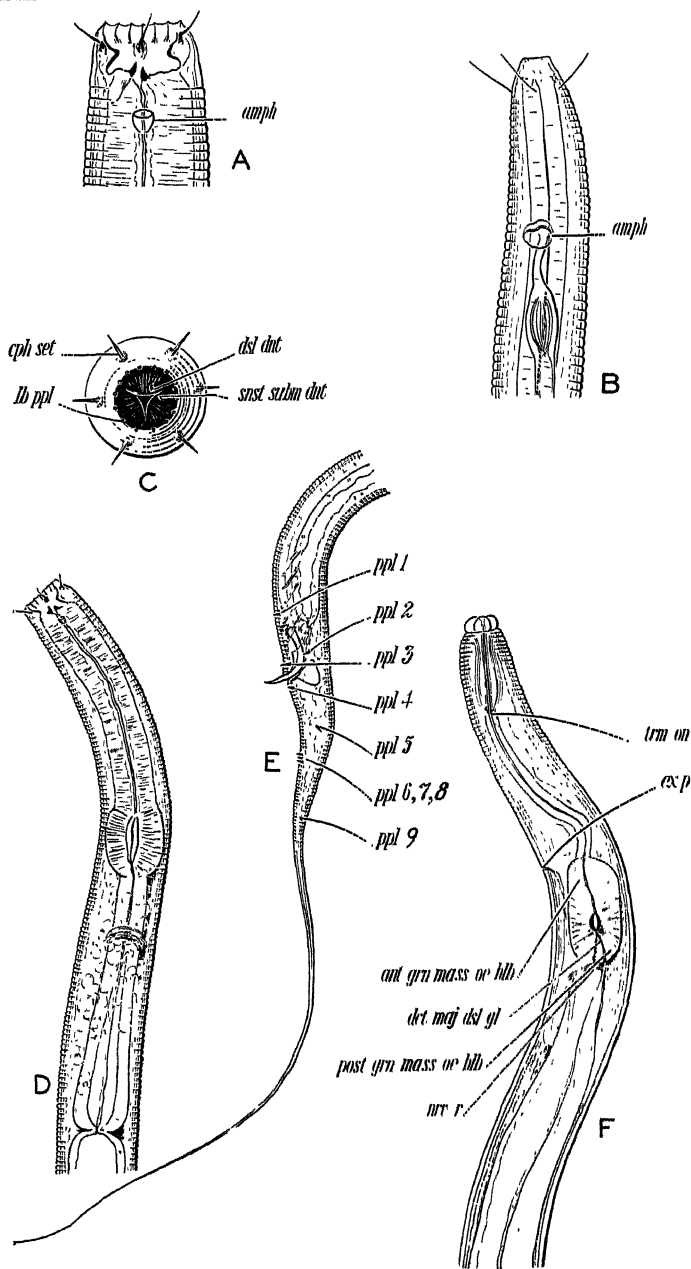


FIG. 10.—A, Head end of *Diplogaster asymmetricus* s. sp.; amph, amphid.  $\times$  about 1,400.  
 B, Head end of *Bastiania longicaudata* De Man; amph, amphid.  $\times$  about 1,400.  
 C, Front view of head of same species; cph set, cephalic setae; dsl dnt, subdorsal onchium; snst subm dnt, left ventro-submedial onchium; lb ppl, labial papillae.  $\times$  about 1,400.  
 D, Anterior end of same species.  $\times$  about 500.  
 E, Tail end of male, same species; ppl 1 to 9, copulatory papillae.  $\times$  about 500.  
 F, Anterior end of *Aphelenchus tenuicaudatus* De Man; ant grn mass or hlb, anterior granular mass of the middle bulb of the esophagus; dct maj dsl gl, ductus of the large dorsal gland; ex p, excretory pore; nrv r, nerve ring; post grn mass or hlb, posterior granular mass of the middle bulb of the esophagus; trm on, posterior end of spear.  $\times$  about 500.

of the cuticle, but it is hard to distinguish the elements of this annulation. These are transverse series of points which seemingly are arranged also in longitudinal series at the conical part of the tail end. The oral opening is wide and surrounded by a membranelike structure which is supported by 14 labial papillae. (Fig. 2, A and B.) These give the walls of the oral cavity a striated appearance when seen from the side. There are six single cephalic setae, each placed on a thickened base. The amphids are located a short distance back of the mouth cavity; the opening is a transverse oval and leads into a cuplike cavity.

On the base of the wide mouth cavity are the two rather small onchia, slightly curved and pointed forward; the side walls of the oral cavity are strong and supported by mobile cuticularized pieces. (Fig. 2, A.)

The cylindrical anterior part of the esophagus back to the middle bulb has radial muscles very well developed, whereas the posterior part consists of an entirely different kind of tissue and gradually swells to form a bulklike end. It seems that this posterior part of the esophagus contains some glandular cells, one of which stands out prominently because of its remarkable nucleus. No excretory pore and no renette cell were seen.

The female sexual organs are double, the ovaries reflexed, and there is in each uterus usually only one egg, which apparently is deposited in an advanced stage of segmentation.

The most remarkable character of the male is its gubernaculum, which, seen from the side, somewhat resembles a flag with a rounded contour and with a staff handle turned toward the anus. Of all the Diplogasters with similar gubernacula, the one of the present species is the largest the writer has ever seen. There are indications that the protrusor muscle of the spicula is attached to the sides of this gubernaculum. A few oblique copulatory muscles were seen in front of the anus.

#### PRISMATOLAIMUS INTERMEDIUS

The only specimen of *Prismatolaimus intermedius* De Man was a female of dwarf size, reaching only 0.045 mm., thus remaining considerably below the minimal size given by various observers. The vulva opened at 62 per cent of the total length. A careful examination of the head end proved that Micoletzky is right in attributing to this species 10 instead of only 6 cephalic setae, confirming also his description and figure of the amphids.

#### BASTIANIA LONGICAUDATA

A single female specimen of *Bastiania longicaudata* De Man (fig. 10, B) was found, with measurements as follows:

0.19	6.2	18	<sup>1457</sup> <sub>15</sub>	90	1.06 mm.
0.58	1.0	1.2	1.9	0.97	

Since the amphids of this species never have been described properly, they are sketched in Figure 10, B. The opening is a somewhat irregular oval, perhaps derived from a former spiral furrow. From it a tube leads inward and backward, somewhat curved, widening farther back to an ampulla, inside of which a number of fine fibers (terminals) could easily be seen; they emerge apparently at the base of this ampulla from an opening and connect here with nerve fibers which go down to the nerve ring.

#### TYLENCHUS INTERMEDIUS

A single specimen of *Tylenchus intermedius* De Man was observed, a female without an egg. It was of very small size, not reaching even the minimal length given by Micoletzky (0.560 mm.). This form is of special interest because of its morphological resemblance to *T. dipsaci*. Its measurements were as follows:

1.6	?	19	?	79	91	0.51 mm.
1.6	?	2.1		1.9	1.6	

## APHELENCHUS TENUICAUDATUS

Measurements of *Aphelenchus tenuicaudatus* De Man (fig. 10, F) were as follows:

	N.R.		End of gland			
Female 1:	4.4	14	15	22	$\frac{370}{3.2}$	$\frac{87}{2}$ 0.58 mm.
	2.5	3.2	3.4	?		
Female 2:	3.6	14	14	35	$\frac{370}{2.7}$	$\frac{90}{1.8}$ 0.62 mm.
	2.1	2.7	2.8	?		

This species was described by De Man (?) in 1895 from *Calanthes vestita* Wall. and *C. veitchii* (the latter said to be a hybrid of *C. vestita* and *C. rosea*) grown in the nursery of Veitch at Chelsea, London, England. De Man gives as measurements for the male 0.8 mm., for the female 0.95 mm., with  $\alpha=35$  to 36,  $\beta$  in the male 8.5 to 9, in the female 9 to 9.5,  $\gamma$  in the male 11 to 15, in the female  $7\frac{3}{4}$  to  $8\frac{3}{4}$ , rarely 10. The specimens were therefore of considerably smaller size than those studied by the Dutch scientist; furthermore, the writer has not seen a male and concludes from this that males must be less numerous than females.

Morphologically, this species is well characterized by its head end with the well-marked six lips; by its long spear, destitute of terminal bulbs (fig. 10, F); by the large medial bulb of the esophagus, which has glandular tissue anteriorly and posteriorly to a remarkable extent; by its extraordinarily long salivary gland, dorsad from the intestine; and by its pointed and elongated tail in both female and male.

The fine annulation of the cuticle has been mentioned by De Man, also the elongated tail, ending in a hairlike portion.

The lip region is well set off; papillae and amphids could not be seen in a side view.

The spear measures 22 to 26 microns and is needle shaped, without bulbs, and without the usual division into an anterior conical and a posterior cylindrical part; it is not even set off from the esophageal tube, but in the living specimens the point of junction between spear and tube can easily be detected, because the spear is stiff, whereas the esophageal tube bends. In the fixed specimens the spear is sometimes very difficult to see, but its inner end can always be detected by the ends of the protruding muscles; one or two fine indications of guiding rings were seen in the wall surrounding the spear. The esophagus, back to the oval bulb, is very slender; the bulb measures 11 to 12 by 20 to 24 microns; it is therefore about twice as long as wide. Nearly its whole anterior half consists of a granular, somewhat glandular substance, penetrated only by the esophageal tube; then follows a muscular section with the central valves, which are rather short and somewhat curved; then follows again a granular terminal portion, through which the esophageal tube connects with the intestine. Apparently connecting with the dorsal side of this granular end portion is the outlet of an extremely long single salivary gland, which extends 142 microns back to the dorsal side of the intestine. In some specimens the outlet of this gland could be seen penetrating the granular end portion of the bulb and emptying into the bulb cavity at the posterior end of the valves. This, however, was rather indistinct. The nerve ring is sometimes located close behind this bulb, in other cases only a short distance caudad.

The anterior end of the intestine is thin walled and transparent, but shows already a remarkably wide cavity, exhibiting therefore a still further advanced transformation of this formerly esophageal part into true intestinal tissue. The walls of the intestine are rather thin, and the cells contain a number of fine globules. The rectum and anal opening in the fixed material are rather obscure, but are readily seen in living material. The excretory pore in the living specimen is mostly in front of the esophageal bulb; in fixed material, however, ventrad from it.

The thin, slender ovaries are always to the right side of the intestine, and the eggs are rather small and apparently deposited in an unsegmented stage. Numerous sperms were seen in a receptacle at the end of the oviduct.

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# THE OCCURRENCE OF ACETALDEHYDE IN BARTLETT PEARS AND ITS RELATION TO PEAR SCALD AND BREAKDOWN<sup>1</sup>

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## INTRODUCTION

The opinion has long been held that functional or physiological disorders of fruits held in storage have their origin in the accumulation of certain toxic metabolites. Several organic substances which may possibly be instrumental in bringing about conditions commonly known as physiological diseases have been identified as products of metabolism. Brooks, Cooley, and Fisher (1),<sup>2</sup> who investigated apple scald, found evidence which suggested that the disease is due to volatile or gaseous substances, other than carbon dioxide, which are produced in the metabolism of the fruit and which can be carried away by air currents or taken up by various absorbents. They were able to produce effects resembling scald by exposing apples to the vapors of various esters (2) and demonstrated that the disease can be controlled by the use of oil-impregnated paper wrappers which are capable of absorbing the odorous emanations from the fruit (3). Power and Chesnut (7) found that the odorous constituents of the apple are the amyl esters of formic, acetic, and caproic acids, with a small quantity of the caprylic ester and a considerable proportion of acetaldehyde, and demonstrated that acetaldehyde is a product of the metabolism of the fruit, suggesting that it might be one of the factors involved in the production of apple scald.

Overholser, Winkler, and Jacob (6), studying the internal browning of Yellow Newtown apples, found that the disease is greatly reduced by ventilating the fruit and, during the early part of the storage season, is likewise reduced by wrapping the apples in oiled paper. They concluded that the disease is due to the accumulation of essential oils or similar deleterious substances which are produced by the apples in storage and that the internal browning of Yellow Newtown apples and apple scald are quite closely related with respect to cause.

Thomas (9) studied the formation of ethyl alcohol and acetaldehyde in apples and found that acetaldehyde was produced in large quantities in certain mixtures of carbon dioxide and oxygen and that high concentrations of this substance were often accompanied by browning of the cells.

The fact that acetaldehyde may occur normally and in relatively large quantities in apple tissues (7, 9) suggests at once the possibility of this substance being intimately related to certain nonparasitic diseases commonly found in stored fruits.

The investigations here reported are the result of a study made upon the occurrence and accumulation of acetaldehyde as a toxic

<sup>1</sup> Received for publication Sept. 30, 1927; issued January, 1928.

<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 992.

metabolite in pear tissue and its relation to the two physiological diseases known as pear scald and pear breakdown.

Pear scald is the name applied to a brown or black discoloration of the outer tissues of the fruit. In early stages the disease is entirely superficial, but it progresses rapidly into the flesh at warm temperatures. The taste and odor of the fruit are characteristically disagreeable and sickening and can be detected in advance of the appearance of actual discoloration.

Pear breakdown or core breakdown is characterized by a brown discoloration and softening of the core tissues. The odor and taste noted in scalded pears is also characteristic of those affected with breakdown, and the two diseases frequently are associated in the same fruit.

Both scald and breakdown have become factors of major economic importance in the handling of pears since the development of modern methods of refrigeration and of the modern commercial canning industry, which involve the handling of pears in large quantities. Scald does not occur on fruit ripened out at ordinary air temperature, but is prevalent on fruit held in cold storage for an extended period, making its most rapid development after removal of the pears to a warm temperature.

## EXPERIMENTAL WORK

### IDENTIFICATION OF ACETALDEHYDE IN PEARS

In order to determine whether acetaldehyde is normally produced in living pear tissue, examination was made of the emanating gases of the fruit without altering the structure of the pears. By so doing, it is reasonably certain that the materials collected were substances resulting from the vital activities of protoplasm, and acetaldehyde, if present, could not possibly be of secondary origin resulting from chemical treatments or extractive agencies.

The method used in this study is a modification of the one described by Power and Chesnut (?). Twenty Bartlett pears were placed in a large glass jar provided with a sealed covering through which two glass tubes were inserted. One of the glass tubes reached to the bottom of the jar, the other to the top level of the pears. The air, which entered the vessel at the top, was first bubbled through a flask containing an alkaline solution of potassium permanganate and then through another flask containing concentrated sulphuric acid. The air passing out of the vessel was bubbled through a flask containing a saturated solution of sodium hydrogen sulphite. Air was drawn slowly through the system by means of an aspirator. This operation was allowed to proceed continuously for six days, at the end of which time the flask containing the sodium hydrogen sulphite solution was detached and the contents were made alkaline with sodium carbonate. This solution was then transferred to a distilling flask and distilled, the condenser and receiving flasks being cooled with ice. The distillate so obtained had the characteristic odor of acetaldehyde, and when subjected to qualitative tests for acetaldehyde the results were always positive and conclusive. With Schiff's reagent a pink color was quickly produced by the addition of a few drops of the distillate. It also reduced an ammoniacal solution of silver oxide, and acetic acid was formed when the distillate was oxidized with

chromic acid. Upon the addition of a few drops of strong sulphuric acid the pink color in Schiff's reagent gradually disappeared, which indicated that formaldehyde was not present. This negative test for formaldehyde was further demonstrated by using the modification of the Rimini test described by Schryver (8), which failed to produce the magenta color which is obtained with very minute quantities of formaldehyde.

The experiments above described present conclusive evidence that acetaldehyde is formed in metabolic processes of pears and that it is emanated in appreciable quantities. Qualitative tests indicate that acetaldehyde is the only aldehyde produced under these conditions, a fact which is in accord with the findings of Thomas (9) and Power and Chesnut (7) with apples.

#### QUANTITATIVE ESTIMATION OF ACETALDEHYDE IN PEAR TISSUES

If acetaldehyde production in pears is associated in any way with physiological diseases, a study of the residual aldehyde content of the tissues would be of importance as being indicative of conditions prior to and accompanying such disturbances. To determine this fact a quantitative study was made of the acetaldehyde in pears showing varying degrees of scald and breakdown.

#### MATERIAL USED

In the following experiments Bartlett pears were used throughout. These were picked August 12, 1926, and were placed immediately in cold storage. This date of picking represented the latter part of the harvest season at Peshastin, Wash., where the fruit for this work was obtained.

Every effort was made to insure uniformity in the selection of material for investigation. In order to reduce to a minimum any variability which might be present in a given lot of fruit, only those pears which were borne on the same tree, harvested at the same time, and stored under the same conditions were used in this work. Samples for analysis were selected on the basis of fruits showing the same visible amounts of scald and breakdown.

In obtaining composite samples for chemical analysis, from four to six pears were used. These were each cut into eight pieces, and samples of tissue from these portions were weighed. After weighing, the tissue was cut into smaller pieces and placed in a 500 c. c. distilling flask. Care was taken to exclude carpel walls and seeds from samples, and all manipulations involving the handling of cut tissues were conducted as rapidly as possible.

#### METHOD OF ANALYSIS

The quantitative method used in these experiments for determining acetaldehyde is a volumetric measure of additive compounds formed when aldehydes combine with sodium hydrogen sulphite. The aldehyde was removed from the pear tissue by steam distillation. About 1,500 c. c. of water was placed in a large round-bottom pyrex flask, through the stopper of which passed a bent glass tube leading to the bottom of the 500 c. c. distilling flask containing the sample of pear tissue. The latter flask was connected with a splash trap and thence to a Liebig condenser, which was cooled with ice water. The delivery tube of the condenser was passed through the stopper

of a large filtering flask and dipped beneath the surface of 50 c. c. of standard sodium hydrogen sulphite solution. The outlet of this filtering flask was connected by a bent glass tube to another smaller filtering flask which also contained 50 c. c. of sodium hydrogen sulphite solution. This second flask was used only as a precautionary measure to retain any aldehyde which might escape from the first flask. Both these receiving flasks were cooled with ice. After the water began to boil vigorously the flask containing the sample was connected to the steam generator and splash trap and the distillation conducted. After 1 liter of distillate was collected, which required about 90 minutes, it was found that all significant traces of acetaldehyde had been expelled from the pear tissue. The quantity of acetaldehyde present in the pear distillate was measured by titrating the sodium hydrogen sulphite in the receiving flasks against standard iodine, adding starch paste as an indicator.

With this method it is essential that control determinations be made and that the iodine and sodium hydrogen sulphite solutions be restandardized each day because of the deterioration of these reagents.

The results of quantitative studies made upon pears for acetaldehyde content are presented in Tables 1 to 7, inclusive. The analyses shown in these tables are representative of a large number of determinations made upon fruits under the physiological conditions then described. Repeated experiments all showed the same trend, but the detailed results are omitted from this paper for the sake of brevity.

#### ACETALDEHYDE IN NORMAL, SCALDED, AND BROKEN-DOWN PEARS

Bartlett pears were removed from cold storage. One lot was sampled immediately and analyzed for acetaldehyde, and those remaining were held for four days in a room where a temperature of 15° to 18° C. was maintained. When these pears were removed from cold storage they were hard, green, and showed no evidence of scalding. After being held for four days at 15° to 18° the fruit began to turn yellow and showed varying degrees of scald, the amount of scald varying from 1 to 10 per cent of the surface of the fruit. Preceding and accompanying the appearance of scald the characteristic disagreeable taste and odor, described in the introduction, was noticeable.

Table 1 shows the quantity of acetaldehyde present in sample tissue taken from the above-mentioned lots, expressed as milligrams of acetaldehyde in 100 gm. of fresh tissue. Composite samples were obtained from six pears of each lot. The acetaldehyde was determined as described under "Method of analysis."

TABLE 1.—*Acetaldehyde content of normal Bartlett pears from cold storage and of similar pears four days after removal from storage*

Sample	Description of sample	Acetaldehyde in 100 gms. of fresh tissue (milligrams)
No. 1.....	Sound green pears from cold storage (no scald).....	0.3
No. 2.....	Pears held for 4 days at 15° to 18° C., about 1 per cent of surface showing superficial scald.	11.9
No. 3.....	Pears held for 4 days at 15° to 18° C., about 10 per cent of surface showing superficial scald.	17.4

The data presented in Table 1 show a rapid increase of acetaldehyde during the four-day ripening period at 15° to 18° C., and during this time scald developed to the extent there recorded.

At the end of the four-day period all fruits in these lots showed more or less scald, so that it was impossible to know whether acetaldehyde would have increased to the same extent in fruits that remained free from scald upon removal from storage.

In order to study this point, an entire box, containing 88 pears, was removed from cold storage. Six pears were taken for immediate sampling and the rest allowed to ripen at a temperature of 15° to 18° C. for a period of seven days. At the end of this time about one-fourth of the lot remained free from scald, whereas the remaining three-fourths showed varying degrees of scald, the severity ranging from slight to complete browning. Samples for acetaldehyde determinations were selected before and after the seven-day period. Analyses of these fruits are given in Table 2.

TABLE 2.—*Acetaldehyde content of scalded Bartlett pears as compared with those free from scald*

Sample	Description of sample	Acetaldehyde in 100 gms. of fresh tissue (milligrams)
No. 1.....	Sound green pears from cold storage (no scald).....	0.3
No. 2.....	Pears ripened for 7 days at 15° to 18° C., remaining free from scald.....	1.8
No. 3.....	Pears ripened for 7 days at 15° to 18° C., showing about 5 per cent of surface scalded.	10.5
No. 4.....	Pears ripened for 7 days at 15° to 18° C., showing about 30 per cent of surface scalded.	19.5
No. 5.....	Pears ripened for 7 days at 15° to 18° C., showing about 100 per cent of surface scalded.*	33.7

\* These pears, in addition to complete scalding, also showed a discoloration of the tissues about the core, typical of pear breakdown already described.

The data from the foregoing tables present an interesting correlation between the accumulation of acetaldehyde and the development of pear scald. In Table 2 this relation is especially striking. Pears which remained free from scald at the end of the seven-day period showed a slight increase in acetaldehyde above that in the ones analyzed immediately after removal from cold storage. However, whenever scald appeared a very marked rise of acetaldehyde occurred, and the concentration of acetaldehyde increased with scald severity.

As is noted above, pears in sample No. 5 showed evidence of breakdown in addition to being completely scalded. In view of the fact that the tissue comprising this sample showed especially large quantities of acetaldehyde, it became important to investigate the presence of acetaldehyde in pears showing breakdown only.

Table 3 presents analyses which are typical of a large number of determinations made upon sound and broken-down pears. In these experiments pears were allowed to ripen at room temperature, about 20° to 22° C., and were sampled when breakdown appeared, usually in about five days after removal from cold storage.

TABLE 3.—*Acetaldehyde content of sound and broken-down Bartlett pear tissue*

Sample	Description of sample	Acetaldehyde in 100 gms. of fresh tissue (milligrams)
No. 1.....	Sound hard pears from cold storage.....	0.3
No. 2.....	Soft ripe pears free from breakdown or scald.....	3.1
No. 3.....	Pears free from scald but showing breakdown at the core.....	23.8

The same general results were obtained in all cases, regardless of whether composite samples from a number of fruits were analyzed or whether analysis was conducted on individual fruits.

Additional confirmation of the evidence presented in Table 3 is to be found in Table 4. In these experiments the pears were removed from cold storage and kept at room temperatures for nine days. At intervals of three days, samples were taken for analysis. Care was taken to discard any fruits showing scald.

TABLE 4.—*Acetaldehyde accumulation in Bartlett pears during a nine-day ripening period at 20° to 22° C.*

Sample	Description of sample	Acetaldehyde in 100 gm. of fresh tissue (milligrams)
No. 1.....	Sound hard pears from cold storage.....	0.3
No. 2.....	Sound soft ripe pears 3 days after removal from storage.....	3.8
No. 3.....	Pears held for 6 days (no scald, but slight breakdown).....	14.1
No. 4.....	Pears held for 9 days (no scald, but badly broken down).....	28.1

The results in Table 4 clearly indicate a relation between acetaldehyde content and the breakdown of pear tissues similar to that which has already been shown to exist between acetaldehyde content and pear scald. These results are in accord with the conclusions of Overholser, Winkler, and Jacob (6) in respect to the common origin of scald and internal browning of Yellow Newtown apples.

If the accumulation of acetaldehyde in the tissues is a causative agent in producing both scald and breakdown of pears, the difference in the two disorders would then be merely one of location in the fruit. To study this further, pears were analyzed to determine the distribution of acetaldehyde in the tissues of fruits having both scalded surfaces and broken-down core areas. Samples were taken from four different regions in the fruit, namely, (1) the scalded skin surfaces, (2) normal tissue adjacent to the skin, (3) normal tissue adjacent to the broken-down core area, and (4) the broken-down core tissue.

The scalded peelings were removed with a paring knife, weighed, and immediately distilled. No effort was made to remove the white, apparently normal, tissue which adhered to the skin. The second sample, which was comprised of normal cortex tissue, was removed at a depth of about 5 mm. around the entire pear. The third sample

also contained only the normal cortex tissue removed from an area about 5 mm. from the broken-down core tissues. In the fourth sample only browned or broken-down tissue was included, and this was carefully separated from the carpel walls before weighing. As in all previous determinations, seeds were excluded from the samples. The distribution of acetaldehyde in scalded and broken-down pears is shown in Table 5.

TABLE 5.—Concentrations of acetaldehyde in localized areas of scalded and broken-down Bartlett pears

Sample	Description of sample	Acetaldehyde in 100 gms. of fresh tissue (milli- grams)
No. 1.....	Scalded parings only.....	18.1
No. 2.....	Sound white tissue of scalded and broken-down pears taken 5 mm. beneath skin.....	15.4
No. 3.....	Sound white tissue adjacent to broken-down core tissue.....	15.7
No. 4.....	Broken-down core tissue.....	24.5
No. 5.....	Parings only from normal pears.....	1.3
No. 6.....	Sound white tissue from normal pears.....	.2

An examination of Table 5 shows that acetaldehyde accumulation takes place at the points of injury, namely, in the skin and in tissues surrounding the core. Although the white, apparently sound tissue showed a fairly high acetaldehyde content, without visible evidence of injury, it is interesting to note that despite the possibility of loss of the volatile acetaldehyde through evaporation or exhalation, it was more concentrated in the scalded tissues of the outer surface of the fruit than in the apparently sound tissues beneath. In the broken-down tissues about the core opportunity for escape of acetaldehyde was much less, and in this region it became most concentrated.

Analyses of sound pears indicated the presence of a very small quantity of acetaldehyde, but, as shown in Table 5, it is more concentrated in the skin tissues than in the cortex.

In each of the preceding experiments acetaldehyde was found in normal as well as injured tissues. Fruits fresh from cold storage had practically the same quantities in all cases. The results shown in Tables 2, 3, and 4 demonstrate that although the pears of sample No. 2 in each of these tables did not develop either scald or broken-down at the end of 7, 5, and 3 days, respectively, there was a slight increase in acetaldehyde content. From this it might be suspected that acetaldehyde may accumulate in the sound tissues of pears that are allowed to ripen with free exposure to the open air. If so, the higher concentrations of acetaldehyde in scalded and broken-down tissues might be attributed to its accumulation in sound tissues until toxic limits are reached.

To demonstrate this possibility, a study was made to determine the change in acetaldehyde taking place in pears from the time they were removed from cold storage until they had completely broken down. For this study, pears were allowed to ripen at room temperatures, 20° to 22° C., and samples were taken for chemical analysis at intervals during this time. Fruits showing visible evidence of scald or fungous decay were carefully excluded from the samples analyzed. The results of this experiment are shown in Table 6,

TABLE 6.—*Acetaldehyde content of Bartlett pears during the period of ripening*

Sample	Description of sample	Acetaldehyde in 100 gm. of fresh tissue (milligrams)
No. 1-----	Sound pears from cold storage.....	0.3
No. 2-----	Pears held for 5 days at 20° to 22° C.....	7.7
No. 3-----	Pears held for 8 days at 20° to 22° C.....	13.3
No. 4-----	Pears held for 10 days at 20° to 22° C <sup>1</sup> .....	23.7
No. 5-----	Pears held for 25 days at 20° to 22° C <sup>2</sup> .....	10.0

<sup>1</sup> All fruits showed core breakdown. <sup>2</sup> All tissues broken down; protoplasm completely disorganized.

From the data shown in Table 6, it is evident that an increase of acetaldehyde accompanies the approach to senescence and reaches its maximum concentration when breakdown of the cells becomes apparent. After 25 days the tissues were completely broken down, and the acetaldehyde content decreased markedly. From the appearance of the fruit it was evident that the cells were dead. The pears were browned or blackened throughout, and when they were broken or cut open the juice exuded freely from the cells. The decrease of acetaldehyde shown in these tissues suggests that these dead cells are either incapable of producing acetaldehyde or unable to retain it.

The progressive accumulation of acetaldehyde in normal pears when freely exposed to the air, as shown in this experiment, is not in agreement with the findings of Thomas (9) with apples. He did not find any appreciable increase of acetaldehyde in apples kept in air either at 1°, 15°, or 22° C. However, in all experiments conducted by the present writers with pears, increase in acetaldehyde accompanied the ripening process. The fact that apples ripen more slowly than pears may account for this difference in results. Pears ripen very rapidly when removed from cold storage and held at warm temperatures. Under these conditions of temperature, respiration proceeds at a rapid rate, and large differences in metabolic products may be measured in a comparatively short time.

In order to study the influence of temperature on the rate of maturing and on the resulting acetaldehyde content of pears, a comparison was made of sound pears taken from cold storage from time to time throughout the investigation. The cold storage referred to is a modern commercial plant, which maintains a temperature of about 0° C., and the temperature did not fluctuate more than 2° C. during the period of the present study. This comparison is presented in Table 7.

It can be seen from Table 7 that pears held at 0° C. did not increase in acetaldehyde content from October 8 to October 30, during which time the ground color remained green. However, as they gradually matured in storage, as indicated in the change of ground color from green to yellow, the acetaldehyde content showed a gradual but significant increase. It can thus be concluded that acetaldehyde is produced in the respiratory activities of pears and accumulates in the tissues as they ripen.

TABLE 7.—Comparison of the acetaldehyde content of Bartlett pears removed from cold storage on different dates

[After December 30, all pears began to show traces of scald]

Date of removal from cold storage	Description of sample	Acetaldehyde in 100 gm. of fresh tissue (milligrams)
Oct. 8.....	Sound pears from cold storage.....	0.3
Oct. 11.....	.....do.....	.3
Oct. 12.....	.....do.....	.3
Oct. 13.....	.....do.....	.2
Oct. 30.....	.....do.....	.3
Nov. 8.....	Sound pears from cold storage (ground color changing from green to yellow).	.7
Dec. 1.....	Sound pears from cold storage.....	1.3
Dec. 13.....	.....do.....	2.5
Dec. 30.....	.....do.....	3.3

The chemical origin of acetaldehyde in living tissues has not as yet been definitely established. However, its close association with ethyl alcohol, as products of alcoholic fermentation (4, 5), suggests that the occurrence of these substances in plant tissues may in part be products of intramolecular respiration. Thomas (9) has demonstrated the presence of a zymase system in apples, this being active even though the air surrounding the apples contains large quantities of oxygen. Acetaldehyde and ethyl alcohol are among the intermediate or end products of this type of intramolecular respiration.

If then, during the life of pears, conditions of environment are such as to stimulate the production of acetaldehyde, toxicity may result after certain limits of concentration have been reached.

The presence of ethyl alcohol in pear tissues will be discussed in a later paper.

#### TOXICITY OF ACETALDEHYDE TO PEAR TISSUES

The toxicity of acetaldehyde to pear tissues can be demonstrated by placing fruit in contact with aldehyde gas. When placed in desiccators, over various concentrations of acetaldehyde, the pears soon develop a brown discoloration which markedly resembles the condition known as pear scald. This browning begins superficially, but gradually penetrates into the cortex until it reaches the core. The length of time required to produce the discoloration, and also the severity of the injury, depend upon the concentration of acetaldehyde to which the pears are exposed. Dilute solutions of acetaldehyde, when injected into the core cavity, cause the tissue in that area to turn brown in color and to have the appearance of typical breakdown.

The term "browning," as used throughout this paper, has reference only to visible results of injury. Browning of the tissues is a secondary action brought about by oxidizing enzymes acting upon injured or dead cells and is produced only after injury or death of the cells has taken place. Therefore, acetaldehyde is merely the indirect cause of the browning.

#### SUMMARY AND CONCLUSIONS

Evidence obtained in the present investigation suggests acetaldehyde as being a possible causative agent in the production of scald and breakdown in Bartlett pears. Although many workers have attrib-

uted the cause of these disorders to toxic products of metabolism, no definite work has been reported associating either pear scald or pear breakdown with the production or accumulation of acetaldehyde within the tissues.

Analyses reported in this investigation show that acetaldehyde is always found in relatively large quantities in scalded and broken-down pears. In these experiments no pears containing more than 14.0 mgm. of acetaldehyde in 100 gm. of fresh tissue, or 0.014 per cent of acetaldehyde by weight of fresh tissue, were found which did not show either scald or breakdown or both.

A positive correlation was found to exist between the severity of scald and the concentration of acetaldehyde in the tissues. A similar correlation was also found between the severity of breakdown in the fruit and the concentration of acetaldehyde in the tissues.

By analysis of pears showing both scald and breakdown, acetaldehyde was found in greater quantities in the scalded skin tissues than in the apparently normal white tissues beneath. The highest quantity, however, was found in the broken-down core areas. This distribution suggests a localization or accumulation of acetaldehyde taking place in two distinct areas, namely, the core and the outer surfaces, and that when the concentrations of acetaldehyde reach a point of toxicity in these areas, browning of the tissues occurs.

That acetaldehyde production is due to metabolic activities and is not necessarily a post-mortem phenomenon was demonstrated by the presence of acetaldehyde in the gases exhaled by sound pears, as well as by increasing quantities found in sound tissues during the period of ripening.

The disagreeable taste and odor preceding and accompanying scald and breakdown are apparently due to acetaldehyde. In extreme cases it was possible to detect a distinct odor of acetaldehyde by simply cutting the affected fruits.

The toxicity of acetaldehyde to pear tissues can be demonstrated by exposing the fruits to acetaldehyde gas or injecting dilute solutions into the tissues. It is possible that this effect may also be brought about with chemical substances other than acetaldehyde, which are present in pear tissues. However, it is doubtful whether these substances ever reach a concentration in pears sufficient to show deleterious effects.

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# WOUND PERIDERM FORMATION IN THE POTATO AS AFFECTED BY TEMPERATURE AND HUMIDITY<sup>1</sup>

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## INTRODUCTION

Largely because of its practical significance, the formation of wound cork in the potato tuber has been the subject of numerous investigations, and at present the factors governing its development are in general well understood. The present investigation had for its primary object the study of the effect of temperature on the suberization process, since the only data available are those by Priestley and Woffenden,<sup>3</sup> who left cut tubers for 12 days at temperatures of 15° and 25° C. and found that the rate of formation may be doubled when the temperature rises from 15° to 25° C.

## MATERIALS AND METHODS

An extensive study of the relation of suberization and wound periderm in cut tubers to infection by *Fusarium* storage rots<sup>4</sup> afforded an opportunity to obtain quantities of material for a study of the effect of temperature and humidity on the wound-healing process. From the cut surface, after various intervals and exposures, tissue blocks were cut, usually about 5 by 15 by 10 mm., with the longest dimension traversing the vascular ring and extending into both cortex and inner phloem. This depth of block is sufficient to include all wound periderm that may be formed below a smooth cut. The material was fixed in ordinary chromo acetic acid and stained with an ammoniacal gentian violet. In preparing the stain it was found that the following procedure gave satisfactory results:

To 70 c. c. of a 1 per cent gentian-violet solution in 80 per cent alcohol add gradually 30 c. c. of aqua ammonia. Stain for several hours or leave the material in the stain over night. Rinse in water and treat for a few seconds with an 8 per cent HCl solution; transfer to water and examine.

It will be found that the suberized cells and old periderm retain the violet stain; the remaining tissue, including the newly formed periderm, becomes colorless.

## SUBERIZATION OF THE CUT SURFACE

The formation of wound periderm, as shown by Appel,<sup>5</sup> and later substantiated by Priestley and Woffenden, is preceded by a process of suberization of the cut surface. The rapidity with which this blocking off by a suberin lamella takes place depends largely on

<sup>1</sup> Received for publication Aug. 8, 1927; issued January, 1928.

<sup>2</sup> This paper reports work carried on while the writer was a member of the Office of Vegetable and Forage Diseases.

<sup>3</sup> PRIESTLEY, J. H., and WOFFENDEN, L. M. THE HEALING OF WOUNDS IN POTATO TUBERS AND THEIR PROPAGATION BY CUT SETS. *Ann. Appl. Biol.* 10: 96-115, illus. 1923.

<sup>4</sup> WEISS, F., LAURITZEN, J. I., and BRIERLEY, P. FACTORS IN THE INCEPTION AND DEVELOPMENT OF FUSARIUM ROT IN STORED POTATOES. [Unpublished manuscript.]

<sup>5</sup> APPEL, O. ZUR KENNNTNIS DES WUNDVERSCHLUSSES BEI DEN KARTOFFELN. *Ber. Deut. Bot. Gesell.* 24: 118-122, illus. 1906.

environmental conditions, especially humidity and temperature. If kept at low humidity the cut surface of the tuber will dry out and crack while the tuber itself shrinks from loss of water; at high humidity, however, suberization will proceed at a rate determined largely by the storage temperature. If the temperature is very low suberization will be greatly delayed, so that noticeable changes will not be observed in the cut surface even after a period of a week or 10 days. Ultimately, however, the surface will suberize. At high humidity and high temperature suberization becomes noticeable even after the first day. It appears first in the outermost exposed cell layer. The walls in this region, as shown by the ammoniacal gentian-violet stain, retain a faint purple color, while the adjacent tissue is hyaline. As suberization progresses the coloration becomes more intense and at the same time the deeper-lying cells begin to retain the stain. The number of affected cell layers does not exceed two or three, and none of the varieties examined show the extreme depth of suberization reported by Priestley and Woffenden for some of their material. Suberization appears to be quite uniform over the entire surface. Wherever vascular bundles are exposed in the cut, the blocking off may extend farther down than elsewhere; but this may be an illusion, since the vascular tissue has a tendency to retain the violet stain, though less distinctly than the suberized cells, if the after treatment with HCl is less prolonged.

The rate of suberization for the Irish Cobbler and Russet Rural varieties is shown in Figures 1 and 2. In the Irish Cobbler variety the beginning of suberization may be seen after the eighth day at  $21\frac{1}{2}^{\circ}$  C. Russet Rural at this time is still unsuberized. At  $5^{\circ}$  suberization in the Irish Cobbler appears after the fifth day and in Russet Rural only after the eighth. At  $10^{\circ}$  in both varieties suberization is observed after the third day, at  $15^{\circ}$  after the second day, and at temperatures between  $21^{\circ}$  and  $35^{\circ}$  after only one day. Increase in temperature from  $21^{\circ}$  to  $30^{\circ}$  appears to have no effect on the rate of suberization, but in several instances at  $35^{\circ}$  suberization was found to extend less deeply. At  $10^{\circ}$  C. and above, the different varieties appear to exhibit no noticeable differences in the rate of suberization.

Olufsen,<sup>6</sup> in his extensive studies on wound periderm formation in the potato, states that moderate moisture is the most essential requirement for periderm formation; an excess of it plays a hindering rôle and causes cell proliferation. At the temperatures here employed no unfavorable effect was observed at the highest humidities, which at the lower temperatures closely approached saturation.

#### FORMATION OF THE WOUND PERIDERM

Since suberization or an equivalent blocking-off process is prerequisite to wound periderm formation, the factors governing the rate of suberization will also affect the second process. No periderm cells were observed at a temperature lower than  $7^{\circ}$  C. within the duration of this experiment. At  $7^{\circ}$  the first periderm cells appeared in the Irish Cobbler variety after the ninth day; in the Russet Rural and other varieties examined no cork cells appeared

<sup>6</sup> OLUFSEN, L. UNTERSUCHUNGEN ÜBER WUNDPERIDERMBILDUNG AN KARTOFFELKNOLLEN. Bot. Centbl., Beihefte 15: [269]-308, illus. 1903.

even on the tenth day. At 10° the first periderm cells appeared in Irish Cobbler after the fourth day, and in the other varieties after the sixth. At 15° periderm cells were seen after the third day, and at 21° and above after the second day, regardless of the variety. Once initiated, periderm formation proceeds rapidly at the higher temperatures, so that after the third or fourth day there are usually present several rows of new cells. (Fig. 3, A.)

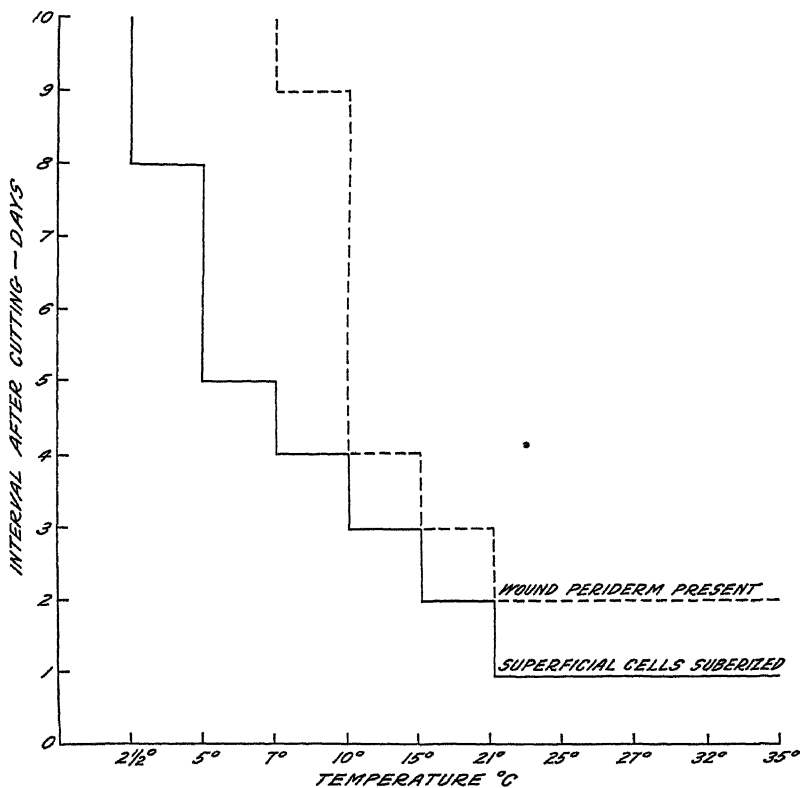


FIG. 1.—Suberization and periderm development in the Irish Cobbler variety

#### EFFECT OF HUMIDITY ON SUBERIZATION AND WOUND PERIDERM

Although at low temperatures variations in relative humidity produce less effect on the evaporation rate than at higher temperatures, an effect on the rate of suberization and periderm development is, nevertheless, noticeable. In tubers of the varieties Green Mountain, Spaulding Rose, and Russet Rural kept at 12° C. for six days at 64 per cent humidity, only the initial stages of suberization became evident; at 74 per cent humidity one or one and a half layers were suberized, and at 94 per cent humidity up to two layers were well suberized. Periderm formation, however, became evident only after the ninth day, and only in the third group, which had been kept at a humidity of 94 per cent.

At lower storage temperatures the effects are similar. At 6.5° C. and 70 per cent humidity, suberization was marked after a period of 53 days, but no periderm developed. At 95 per cent humidity, however, a well-developed periderm was noticeable. Different tubers often react differently. For instance, tubers of the Green Mountain variety, kept under identical conditions, showed the presence of a wound periderm in one case and its absence in another. No explanation can yet be offered for such irregularities, but their relation to infection of injuries despite apparently favorable conditions for healing is suggestive.

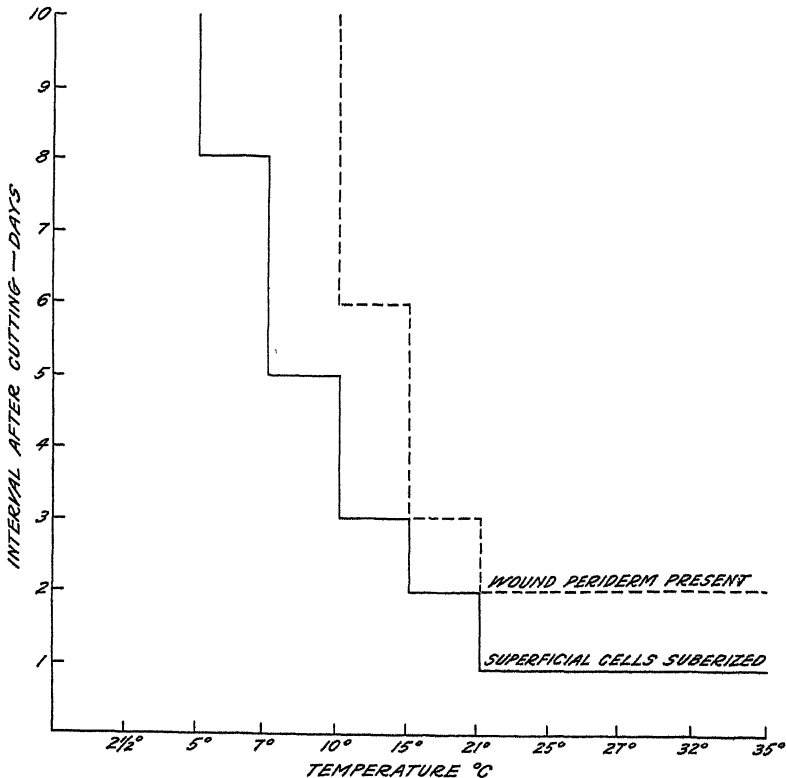


FIG. 2.—Suberization and periderm development in the Russet Rural variety

In a cross section of the tuber the topography of the periderm surface is practically flat; it may extend slightly nearer the wound surface in the vascular ring, but the difference is insignificant. The central region may sometimes show a marked contrast, exhibited in fewer periderm layers which lie farther from the surface, and this is especially true if the central medulla is very large. If the medulla is small, that is, if the phloem groups extend practically to the center of the tuber, no such regional differences will be observed. When the xylem vessels are transected, the wound periderm layer is formed around but not through them. The vessels are, however, subject to blocking by deposits of gum, which extend but a short distance back from the cut.

Concomitant with periderm formation is the disappearance of starch from the cell layers next to the cut surface. It is of interest to note, however, that the protein crystals, found abundantly in the outer cortical cells, are not used up during this process.

#### EFFECT OF TYPE OF INJURY ON WOUND PERIDERM

While suberization and wound cork formation in cut and stab wounds follow the method given above, tubers from which the skin

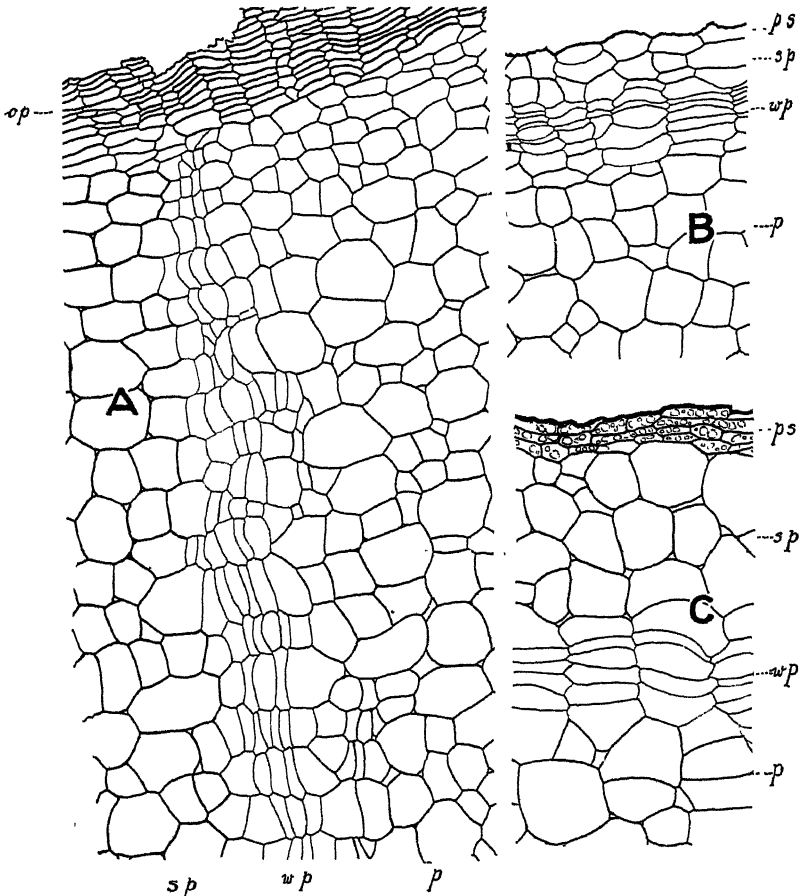


FIG. 3.—A, Wound cork formation in the Early Rose variety: *op*, old periderm; *sp*, suberized parenchyma at surface of cut; *wp*, wound cork; *p*, storage parenchyma.  $\times 85$ . B, Wound cork formation in the Early Rose variety; tuber was injured by peeling off skin; peel removed smoothly: *ps*, peeled surface; *sp*, suberized parenchyma; *wp*, wound periderm; *p*, storage parenchyma.  $\times 85$ . C, Same as B, except that in peeling the surface was much abraded. Note that wound periderm is formed farther away from injured surface.  $\times 85$

is peeled or which are injured by contusion show a different behavior. When young tubers are injured by blows, the young periderm cells are commonly crushed, while the older periderm shows little effect. (Fig. 4, C.) Typically there is an irregular development of periderm below the margin of the injury or wherever there is an actual break in the original periderm, but there is only slight or no development of periderm beneath the tissues which are merely crushed. (Fig. 4,

A.) It may happen that the wound periderm is formed in several tiers with crushed necrotic parenchyma cells intervening.

Tubers injured by peeling off the skin respond readily with the development of an extensive periderm. When the peel is removed

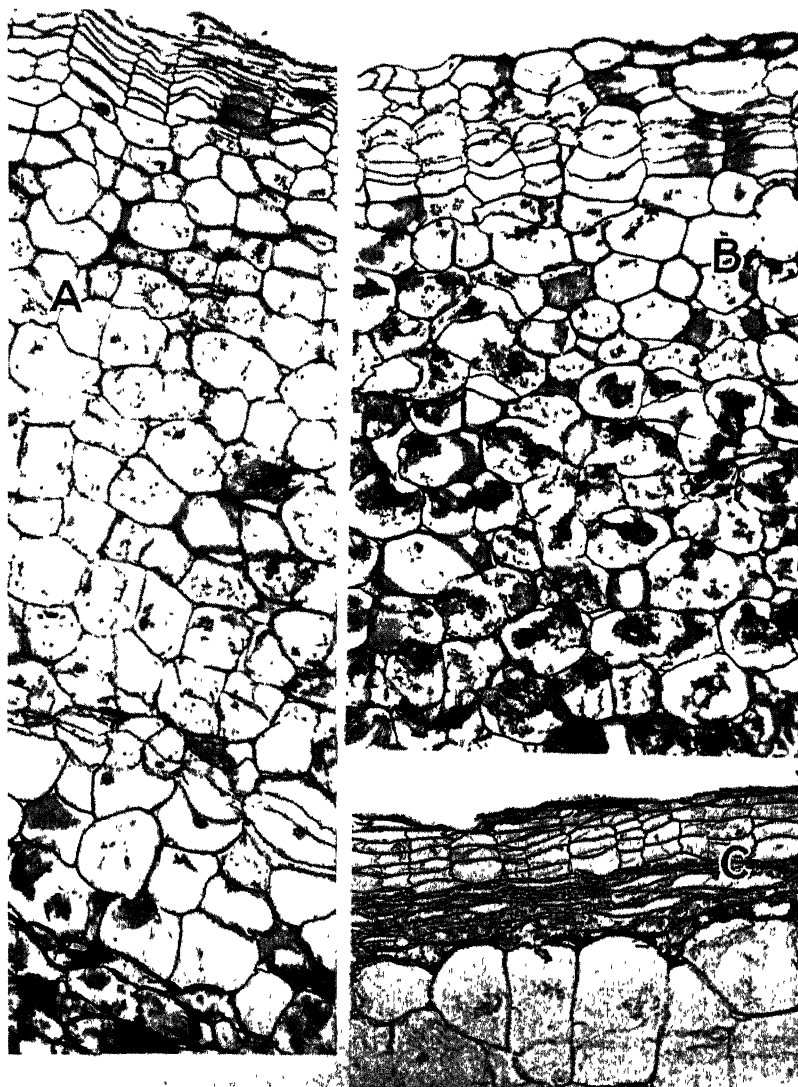


FIG. 4.—A, Deep-seated wound periderm formation in tuber injured by blows.  $\times 85$ . B, Superficial wound cork formation in tuber injured by peeling off the skin smoothly.  $\times 85$ . C, Young tuber injured by blows; the young periderm cells are crushed, whereas the old periderm cells are not harmed.  $\times 85$

smoothly, the new periderm cells develop within one cell layer of the surface (fig. 3, B, and fig. 4, B), whereas if the surface is much abraded, a deeper-seated periderm results (fig. 3, C).

# SOME RELATIONS OF THE PHOSPHOLIPINS IN SEEDS TO OTHER CONSTITUENTS<sup>1</sup>

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## INTRODUCTION

Since phosphorus containing lipid substances is so universally present in plant tissues, and is so commonly found in those parts of plants in which the vital phenomena are most pronounced, it is evident that it plays an important rôle in the life processes of those tissues. Many theories have been put forward to explain its function. Overton (22),<sup>3</sup> for example, in 1901, presented the theory that each living cell is surrounded by a semipermeable membrane consisting of lipid material, which regulates the passing into and out of the cell of substances necessary for metabolism and growth. However, present knowledge of colloidal substances indicates that Overton's lipid membrane is not essential to a proper explanation of the migration into and out of the cell protoplasm of nutritious substances.

Other investigators have cited experimental results which appear to indicate that these lipid substances play a very important rôle in the process of fat metabolism. Bang (5) has pointed out that the lipoids are probably the most labile of all the constituents which compose the colloidal system known as plant protoplasm, and, by virtue of this property, play an exceedingly important part in the metabolic processes. The same labile character which apparently makes them so important in the chemical changes in the plant tissue makes them equally unstable compounds with which to work. For this reason pure preparations for investigational purposes are very difficult to obtain, and as a result there is as yet no definite and certain knowledge of what their functions are.

## REVIEW OF LITERATURE

The presence of phospholipins in plants was probably first observed by Knop (18), who isolated from certain plant tissue fatty extracts which contained phosphorus. This observation was confirmed the following year by Töpler (24). A few years later Hoppe-Seyler (15) isolated from peas a fatty substance very similar in nature to egg lecithin. Heckel and Schlagdenhauffen (14) showed that the phospholipins were of general occurrence in plants. The first definite proof of the similarity of these substances to lecithin was given by Jacobson (17), who showed that one of the decomposition products of these phospholipins was choline. Shortly afterwards Schulze and

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<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 1017.

Likiernik (27) obtained from seeds lecithinlike substances, which, when hydrolyzed, gave fatty acids, glycerophosphoric acid, and choline, and for a time it was believed that the only phospholipin present in plants was lecithin. But further investigation soon revealed the presence of phospholipins other than lecithin.

The investigations along this line have consisted essentially in the extraction of the fatty substances from the plant tissues with various solvents and estimating the phosphorus in the fatty extract. The amount of lecithin present was calculated from the phosphorus thus obtained. Töpler (24) extracted several varieties of seeds with ether and estimated the percentage of lecithin by this method. A few years later Beyer (7) and Jacobson (17) demonstrated that ether was unsuitable for the quantitative extraction of these substances. Schulze and Steiger (28) showed that more complete extraction could be obtained by using both ether and alcohol in the extraction process. A number of quantitative estimations were carried out, using the ether-alcohol extraction method, by Schulze and Steiger (28), Bittó (9), Stutzer (30), and others (31). The accuracy of such a method would necessitate the complete extraction of the phospholipins, and the extraction of no other phosphorus-containing substance. Bittó (8) criticized Schulze and Steiger's ether-alcohol extraction on the basis of incompleteness. Schulze (26) maintained that Bittó's criticism had but little weight, since materials which had been extracted by this method retained only traces of lipid phosphorus. Later (25) he stated that it was not certain that cold alcohol would completely extract the phospholipins from plant material, but to guard against decomposition he recommended the maintenance of the extraction temperature under 60°. From time to time other investigators have adopted slight modifications of the above method. Results obtained by these methods give but a general idea of the phospholipin content of the substances examined, and are not to be regarded as absolute.

While no perfectly reliable method for the estimation of the plant phospholipins has as yet been evolved, results obtained by different investigators indicate that the leguminous seeds contain considerably more of these substances than do the cereal grains.

A number of attempts have been made to establish a relationship between the amounts of phospholipins in seeds and some of the other constituents. Schulze and Likiernik (27) and Schulze, Steiger, and Maxwell (29) found that the phospholipins varied directly with the protein content. This observation was later confirmed by other investigators. Parrozzani (23) made observations which led him to believe that the ratio is more definite with the amide nitrogen than with the total protein.

Thus it is apparent that the available information concerning the plant phospholipins is very meager, especially that which relates to function in the life processes of the plant.

The investigations herein reported were made for the purpose of (1) ascertaining the relative amounts of phospholipins in various seeds and determining the relation of the phospholipins to the ash, protein, fat, and total phosphorus present in the seeds; (2) to ascertain the relation of the phospholipin content to the ash, fat, protein, and phosphorus content at different stages in the development of the seeds; and (3) to ascertain the effect of germination on the com-

position of the seeds. The investigations were applied to the seeds of the grain sorghums in particular, since this group offers a closely related series when considered from the botanical standpoint, yet one whose members differ in color, size, chemical composition, and nutritive properties.

#### EXPERIMENTAL METHODS

For the first group of experiments 60 samples of seeds, representing different varieties of corn, beans, peas, wheat, grain sorghums, and others were secured from the agronomy department. The only data available concerning these seeds were the name of the variety and the year in which it was grown. The seeds when obtained were clean, dry, and in a good state of preservation, being free from molds, weevil, dirt, and other contaminations. From 150 to 200 gm. portions of each variety were ground in a small hand mill, until the total material passed through a 60-mesh sieve. After grinding, the samples were exposed to the atmospheric conditions of the laboratory for a time, mixed thoroughly, and preserved in glass containers. Moisture determinations were made upon each sample previous to the analysis. This determination was made by drying a 2 gm. sample to constant weight in an electric oven at 100° to 102° C.

The ash determination was made by incinerating a 2 gm. sample to dull red heat in an electric muffle. Nitrogen was determined by the Gunning modification of the Kjeldahl method. Protein was calculated by multiplying the nitrogen thus obtained by the conventional factor 6.25. The use of this factor induces a source of error, as it has been found that it varies with the different seeds. But since the factors for all the seeds involved have not been definitely established, and since most of the available data concerning the protein of these seeds have been obtained by this factor, it was used in this investigation. The fat, more properly termed "ether extract," was determined by extracting a 2 gm. sample of the dry material with anhydrous ether in a continuous extractor for 16 hours and noting the loss in weight of the sample.

The total phosphorus was estimated according to the method of the Association of Official Agricultural Chemists for the estimation of phosphorus in plant constituents (4). The results are expressed as phosphorus in order to permit a more direct comparison with the results obtained in the phospholipin determination.

The phospholipins were estimated by extracting the dry seeds with an ether-alcohol mixture, and estimating the phosphorus in the extracted material by the microcolorimetric method (12). The material was dried in an electric oven for two hours at a temperature of 100° to 102° C. before weighing out the sample.

Several modifications of the former procedure were made in accordance with the type of seeds under investigation. The amount of solvent varied from 50 to 250 cubic milliliters per gram of material, depending upon the fat content of the particular seed. After the tightly corked extraction tubes had been shaken vigorously for 10 minutes, they were placed in a warm bath maintained at 30° C., where they remained for two hours. At the end of this time the tubes were again shaken and allowed to settle. Phosphorus was determined in aliquots of the supernatant liquid. This determination

was performed in sets of 10 each, and usually three or four standards of different concentration of phosphorus were carried along simultaneously. No comparisons were made in which the standard differed more than 30 per cent from the unknown solution. For example, when a standard was set at 20, the reading of the unknown fell between the limits of 14 and 26. The results are expressed as percentage of phosphorus in the dry materials.

For ease of study and interpretation, the results of the analyses, when possible, have been grouped according to the various types of seeds and presented in table form. All determinations were made in duplicate or triplicate, and repeats made until consistent agreements were obtained. The data presented are the average values obtained by this method.

#### EXPERIMENTAL DATA

##### RELATION OF THE PHOSPHOLIPINS TO THE ASH, PROTEIN, FAT, AND TOTAL PHOSPHORUS IN VARIOUS SEEDS

The constituents of the grain sorghum seeds determined on a dry-matter basis are shown in Table 1. In considering the data here presented, a considerable variation in the percentage of the several constituents is noted. These variations are not limited to the different varieties of seeds, but occur in different samples of the same variety. Such findings indicate that the composition of a seed depends, at least to some extent, upon factors other than type and variety. It is common knowledge that both soil and climatic conditions have a definite effect upon the ash, protein, and phosphorus content of many seeds. The effect of these factors upon the fat and phospholipin in a given type of seed have not as yet been definitely established, but it stands to reason that these two components would be augmented, more or less, by the same factors. If the variations in soil composition and climatic conditions do not explain the discrepancies in the data presented, then we have no explanation to offer.

A number of investigators have determined the percentage of lipid phosphorus in various seeds, multiplied these values by a factor, and designated the products as the percentage of lecithin in the seeds. Such a procedure must be based upon two assumptions: (1) That lecithin is the only phospholipin in such seeds, and (2) that lecithin has a constant composition. Both of these assumptions have been proved to be false, for phospholipins other than lecithin are known to exist in seeds, and purified lecithin from various seeds has been found to contain various percentages of phosphorus. This variation is readily understood when we consider the complex molecular structure of this substance and the possibility of its forming conjugated products with other substances. This discrepancy in the percentage of phosphorus may be explained in several ways. First, by any variation in the molecular weight of the fatty acids which compose the lecithin molecule. Any increase in molecular weight would result in a decrease in the percentage of phosphorus, while a decrease in molecular weight would have the opposite effect. Such variations in the fatty acids are frequently found in the more familiar glycerol esters or fats. For this reason, this type of phospholipins should not be classed as a single substance but as a class of substances having many possible members. A second explanation of this devia-

tion may be found in the fact that the phospholipins are frequently found conjugated with other substances, which results in a marked increase in molecular weight and consequently a decrease in the percentage of phosphorus. For example, a number of investigators, on finding their purified product to contain less than the theoretical percentage of phosphorus, subjected it to hydrolysis and obtained therefrom one or more molecules of carbohydrate. A third explanation lies in the fact that the complex substances such as the phospholipins are exceedingly difficult to purify and yet more difficult to preserve in a stable form after purification. Consequently the variation in the phosphorus content may be due to contamination or to products of disintegration of the originally pure substance. The contaminating substance may be phospholipin of different molecular structure than that of the lecithins, or it may be a nonphosphorus-containing substance.

TABLE 1.—Percentage of ash, protein, fat, total phosphorus, and lipid phosphorus in seeds of the grain sorghums, determined on a dry-matter basis, 1924 to 1926

Seeds used	Sample No.	Year	Ash	Protein	Fat	Total phosphorus	Lipid phosphorus
			<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Darso	1	1924	1.87	12.56	3.12	0.240	0.0310
	20	1925	2.00	11.94	3.35	.227	.0353
	66	1926	1.53	13.28	3.70	.258	.0392
	72	1926	2.12	11.88	2.76	.249	.0372
Red kafir	2	1924	1.72	12.66	3.21	.183	.0420
	18	1925	1.87	9.56	2.24	.179	.0436
	64	1926	1.55	11.72	3.24	.201	.0452
	3	1924	1.88	12.44	3.59	.214	.0330
White kafir	12	1925	1.74	12.35	2.73	.205	.0456
	73	1926	1.81	12.15	3.11	.218	.0356
	4	1924	2.12	12.37	3.08	.253	.0360
White milo	16	1925	2.22	12.06	2.39	.214	.0370
	5	1924	1.68	12.94	3.73	.288	.0260
	13	1925	1.75	12.50	2.67	.310	.0356
Feterita	67	1926	1.63	14.47	2.71	.323	.0356
	15	1925	2.17	10.83	2.12	.253	.0420
	71	1926	2.29	10.50	2.53	.266	.0454
Yellow milo	74	1926	2.14	12.56	2.90	.253	.0505
	14	1925	1.85	11.94	2.36	.214	.0372
	17	1925	1.94	13.91	2.74	.192	.0328
Sorgo	70	1926	1.60	11.25	3.10	.227	.0336
Reeds kafir	19	1925	2.49	12.65	3.35	.210	.0396
Sorghum	21	1925	1.72	9.19	2.70	.201	.0356
Common kafir	65	1926	1.57	12.66	3.02	.340	.0360
Hegari	68	1926	1.26	12.94	3.53	.297	.0364
Sunrise kafir	69	1926	1.64	11.75	3.37	.345	.0354

\* This variety was not identified.

Thus it is apparent that any attempt to express the phosphorus-containing lipid substances extracted from seeds or any other organic tissue as a single substance is without justification. Consequently, in this investigation the lipid phosphorus extracted from the seeds has been estimated and this value has been designated as phospholipins. Lipid phosphorus and phospholipins are to be considered synonymous so far as this investigation is concerned. It is true that the two terms designate entirely different substances, but in this case they bear a direct relationship to each other. And since this relationship has not been definitely established for the various phospholipins nothing can be gained by multiplying by an arbitrary factor, and a higher degree of inaccuracy results.

Considering the above facts, the values submitted are not to be considered as absolute but as indices of the relative amounts of phospholipin in these seeds.

It was thought that the phospholipin content of a given type of seed is always constant, regardless of the several factors which might affect the seeds during growth. However, this does not appear to be the case when the values obtained from the 26 samples of grain-sorghum seeds are considered.

Since the actual amounts of these substances in the seeds examined are comparatively small, the magnitude of the variations becomes apparent only when the percentage variation between the different values is considered. When this is done the percentage variation between the maximum and minimum values is found to range from 0 to about 96 per cent—a fact which is unexplainable at the present time. The phospholipin of this class of seeds does not appear to bear any definite relationship to any of the other constituents estimated.

Table 2 gives the analysis of six varieties of corn. It is to be noted that the phospholipin content of these six samples, while fairly constant, is much less than that in the grain-sorghum seeds. This fact indicates at once that the phospholipin content of seeds is at least not always proportional to the protein content. And since the fat content of the corns is higher than that of the grain sorghums, there is no apparent relationship between phospholipin and fat content, at least in this particular case. Phospholipin content appears to be independent of the color of the seeds examined.

TABLE 2.—Percentage of ash, protein, fat, total phosphorus, and lipoid phosphorus in seeds of corn of different varieties; determinations on air-dry matter, 1925

Variety	Sample No.	Ash	Protein	Fat	Total phosphorus	Lipoid phosphorus
		<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Reids Yellow Dent.....	22	1.53	12.18	4.63	0.214	0.0104
Silvermine.....	23	1.29	10.28	4.12	.205	.0142
Gehu.....	24	1.54	14.28	4.24	.201	.0136
St. Charles White.....	25	1.16	10.18	3.91	.183	.0134
Bloody Butcher.....	26	1.35	10.47	4.48	.196	.0145
Pop corn.....	48	1.60	11.50	4.75	.179	.0143

Table 3 contains the analysis of five samples of at least four varieties of wheats. The composition of this class of seeds resembles that of the grain sorghum seeds in many respects.

TABLE 3.—Percentage of ash, protein, fat, total phosphorus, and lipoid phosphorus in seeds of wheat of different varieties; determinations on air-dry matter, 1925

Variety	Sample No.	Ash	Protein	Fat	Total phosphorus	Lipoid phosphorus
		<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Kanred.....	38	1.93	11.94	2.26	0.314	0.0321
Harvest Queen.....	39	1.81	12.50	1.81	.262	.0332
Turkey Red.....	40	1.88	11.38	2.34	.279	.0312
Black Hull.....	41	2.03	14.10	2.19	.310	.0291
Wheat?.....	10	1.74	12.35	2.24	.279	.0272

Table 4 contains the analysis of a number of different varieties of beans. It will be readily observed that the percentage of phospholipins in this class of seeds is very much higher than in any of the seeds previously mentioned. This high phospholipin content appears to bear some relation to the high protein content of these seeds. No similarity can be observed between the phospholipin and either the ash or total phosphorus content.

TABLE 4.—*Percentage of ash, protein, fat, total phosphorus, and lipid phosphorus in beans of different varieties, determined on a dry-matter basis, 1925*

Variety	Sample No.	Ash	Protein	Fat	Total phosphorus	Lipid phosphorus
		<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Mung bean.....	6	4.90	26.12	11.13	0.389	0.1050
Soy?.....	7	4.93	35.25	18.42	.445	.1580
Soy Midwest.....	34	5.25	35.91	23.12	.389	.1025
Soy Manchu.....	35	4.80	38.08	22.66	.445	.0968
Soy Wilson.....	27	3.74	32.78	17.82	.332	.0944
Soy Morse.....	33	4.98	35.26	9.46	.419	.0780
Velvet Georgia.....	36	2.66	24.35	6.05	.402	.0832
Velvet Tracys.....	37	2.88	24.53	5.10	.389	.0834
Velvet?.....	8	2.52	28.10	10.22	.419	.0790
Navy bean.....	49	3.48	23.85	1.85	.244	.0452

The analysis of peas of five varieties is shown in Table 5. The composition of this type of seeds appears to be very constant, so far as the several constituents determined are concerned. The percentage of phospholipin present is slightly less than that found in the bean family, but is much greater than that in either grain sorghum, corn, or wheat.

TABLE 5.—*Percentage of ash, protein, fat, total phosphorus, and lipid phosphorus in peas of different varieties, determined on a dry-matter basis, 1925*

Variety	Sample No.	Ash	Protein	Fat	Total phosphorus	Lipid phosphorus
		<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Black eye.....	28	3.21	25.60	2.02	0.266	0.0784
Whippoorwill.....	29	3.59	25.81	1.56	.314	.0733
New Era.....	31	3.49	29.40	2.20	.279	.0784
Scotch.....	30	3.23	24.41	1.45	.288	.0723
Golden Vine.....	32	3.70	29.87	1.17	.310	.0776

Table 6 contains the percentage of the various constituents in a number of miscellaneous seeds. The point of interest in this table is the fact that cottonseed, which contains the greatest amount of phospholipin, shows less protein and less fat than the peanut seed. This again reminds us of the difficulty encountered in attempting a correlation between the various components of a given seed.

TABLE 6.—Percentage of ash, protein, fat, total phosphorus, and lipoid phosphorus in several different kinds of seeds, determined on a dry-matter basis, 1925

Seeds used	Sample No.	Ash	Protein	Fat	Total phosphorus	Lipoid phosphorus
		<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Peanut.....	9	2.15	33.03	48.65	0.332	0.0880
Cottonseed.....	11	4.78	23.50	25.20	.476	.1456
Hanna barley.....	42	2.08	13.12	1.80	.275	.0442
Russian Green oats.....	43	2.15	13.06	4.27	.266	.0338
Kanota oats.....	44	2.31	13.56	3.83	.258	.0348
German millet.....	45	4.34	10.87	3.32	.157	.0168
Timothy.....	46	5.34	15.60	3.63	.332	.0431
Clover.....	47	4.32	35.19	5.42	.271	.0506

Table 7 contains the average composition of the several types of seeds examined, arranged in the order of increasing phospholipin content. It is evident from this table that there is no relationship between phospholipin and ash content of various seeds. There appears to be a slight correlation between the phospholipin content and each protein, fat, and total phosphorus content of the various classes of seeds examined. This correlation, while more striking between the protein content than between the other constituents, does not appear to be always present. A further attempt at such a correlation should be made on a larger number of samples of different types of seeds grown upon the same type of soil and under similar climatic conditions. Such an investigation would eliminate two variable factors, which without a doubt have had a profound effect upon the results obtained in this group of experiments, namely, soil and climatic conditions.

TABLE 7.—Average composition of the several types of seeds examined arranged in the order of increasing phospholipin content

Seeds used	Number of samples examined	Number of varieties examined	Composition				
			Ash	Protein	Fat	Total phosphorus	Lipoid phosphorus
			<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Corn.....	6	6	1.42	11.47	4.34	0.196	0.0144
Millet.....	1	1	4.34	10.87	3.32	.157	.0168
Wheat.....	5	4	1.88	12.45	2.17	.280	.0806
Oats.....	2	2	2.23	13.31	4.05	.262	.0368
Grain sorghums.....	26	12	1.85	12.12	2.98	.245	.0378
Timothy.....	1	1	5.34	15.60	3.63	.332	.0431
Barley.....	1	1	2.68	13.12	1.80	.275	.0442
Clover.....	1	1	4.32	35.19	5.42	.271	.0506
Peas.....	5	5	3.44	27.02	1.68	.292	.0760
Peanut.....	1	1	2.15	33.03	48.65	.332	.0880
Beans.....	10	8	4.01	30.42	12.59	.387	.0926
Cottonseed.....	1	1	4.78	23.50	25.20	.476	.1456

#### RELATION OF THE PHOSPHOLIPINS TO THE ASH, PROTEIN, FAT, AND TOTAL PHOSPHORUS IN SEEDS OF GRAIN SORGHUMS AT DIFFERENT STAGES OF MATURITY

The investigations of Schulze and Frankfurt (26) led them to believe that unripe seeds contained much less phospholipins than ripe seeds. On making a study of the composition of corn and bean seeds at different stages of growth, André (1) found that the total nitrogen

in the dry matter increased as maturity approached, but the percentage of both ash and nitrogen decreased. André (2) found also that the amount of ash in lupine and haricot seeds increased with maturity, but the actual percentages decreased, while there was a definite increase in both the amount and percentage of phosphoric acid. Further investigations by André (3) led him to believe that the transformation of nitrogen during maturation is the reverse of that during germination. Zaleski (34) observed an increase in protein phosphorus with a corresponding decrease in phosphoric acid as the seeds approached maturity. The lipoid phosphorus remained practically unchanged. McClenahan (20) made a very complete study of the fatty substances of the black walnut at various stages of growth and the results obtained indicated a definite decrease in the phospholipin content with the approach of maturity.

From the examples cited, it is evident that there is a lack of agreement concerning the effect of the stage of maturity upon the phospholipin content of seeds. No satisfactory explanation of these discrepancies is available. One possible explanation is that the variation in the phospholipin of seeds at successive stages of growth differs with the particular seeds investigated. Analyses were therefore made to ascertain the relation of the phospholipin content of the seeds of certain members of the grain-sorghum family to the ash, fat, protein, and phosphorus content of the respective seeds at various stages of development.

Twenty-one samples, representing seven varieties of grain sorghums, were used. These samples were obtained from the agronomy department and had been grown simultaneously upon adjoining plots, on similar soils, and under similar climatic conditions. The three stages at which the seeds were gathered are designated as milk, dough, and mature. Since the head of the grain-sorghum plant does not ripen uniformly from base to apex, only such portions of each head were selected as would fall in each of these stages. Thus, samples were obtained which better represented the different stages of maturity of the different varieties than could have been procured by taking the whole heads of seeds from fewer plants. As soon as the samples were collected they were taken to the laboratory where they were freed of hulls, stems, and other adhering material. Two 100 gm. portions of each sample were weighed, placed in glass containers without tops, and dried at room temperature. When dry, the seeds were ground in a small hand mill until the total mass passed through a 60-mesh sieve, and then stored in glass containers until the desired analysis could be made.

To ascertain the moisture content of the freshly picked seeds, duplicate 10 gm. samples were placed in moisture dishes and dried to constant weight in an electric oven at 100° C. It was found that the average percentage of moisture in the fresh seeds in the milk, dough, and mature stages was 52, 40, and 29, respectively. The moisture content of each sample of the air-dry material was determined just before the analysis.

The procedure and methods of analysis were the same as those employed in the first group of experiments.

The results of the analyses have been arranged in tabular form to facilitate comparison. (See Tables 8 and 9.) The figures given

represent the average values obtained by two or more consistent determinations. Table 9 contains the same data as Table 8, except that the determinations have been made on the basis of the original material.

TABLE 8.—Percentage of ash, protein, fat, total phosphorus, and lipid phosphorus in sorghum seeds at different stages of maturity, determined on a dry-matter basis

Seeds used	Sample No.	Stage	Dry matter	Ash	Protein	Fat	Total phosphorus	Lipoid phosphorus
			<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Red kafir	50	Milk	42.0	2.43	11.78	2.36	0.156	0.0261
	57	Dough	56.5	1.87	11.32	2.87	.182	.0300
	64	Mature	74.2	1.55	11.72	3.24	.200	.0452
Common kafir	51	Milk	50.0	2.11	12.07	1.91	.286	.0300
	58	Dough	66.5	1.71	12.00	2.46	.317	.0328
	65	Mature	70.5	1.57	12.65	3.02	.343	.0360
Darso	52	Milk	46.5	2.54	11.82	1.50	.129	.0271
	59	Dough	57.8	1.97	13.28	2.57	.190	.0284
	66	Mature	72.5	1.53	13.28	3.70	.255	.0302
Spur feterita	53	Milk	53.4	2.52	13.75	1.86	.238	.0198
	60	Dough	58.1	1.89	13.78	1.94	.247	.0217
	67	Mature	72.0	1.63	14.47	2.71	.323	.0356
Hegari	54	Milk	42.6	2.60	11.41	1.66	.247	.0219
	61	Dough	51.2	1.82	12.66	2.66	.249	.0261
	68	Mature	70.2	1.25	12.94	3.53	.299	.0364
Sunrise kafir	55	Milk	54.2	3.09	10.22	1.69	.251	.0256
	62	Dough	60.5	2.15	10.57	2.51	.317	.0274
	69	Mature	72.5	1.64	11.75	3.37	.347	.0354
Reeds kafir	56	Milk	48.5	2.74	10.32	1.88	.172	.0221
	63	Dough	64.2	1.88	10.44	2.96	.212	.0283
	70	Mature	68.5	1.60	10.50	3.10	.229	.0336

TABLE 9.—Percentage of ash, protein, fat, total phosphorus, and lipid phosphorus in sorghum seeds at different stages of maturity, determined on the fresh material

Seeds used	Sample No.	Stage	Factor	Ash	Protein	Fat	Total phosphorus	Lipoid phosphorus
				<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Red kafir	50	Milk	0.420	1.02	4.95	0.99	0.065	0.0110
	57	Dough	.565	1.05	6.31	1.61	.012	.0168
	64	Mature	.742	1.15	8.67	2.40	.118	.0334
Common kafir	51	Milk	.510	1.06	6.01	.97	.143	.0150
	58	Dough	.665	1.13	7.92	1.62	.209	.0216
	65	Mature	.705	1.10	8.86	2.11	.240	.0253
Darso	52	Milk	.465	1.17	5.44	.69	.059	.0125
	59	Dough	.578	1.14	7.70	1.49	.110	.0147
	66	Mature	.725	1.10	9.56	2.66	.184	.0282
Spur feterita	54	Milk	.534	1.35	7.34	.99	.127	.0106
	60	Dough	.581	1.12	7.99	1.49	.143	.0126
	67	Mature	.720	1.17	10.42	1.95	.233	.0256
Hegari	54	Milk	.426	1.11	4.86	.71	.105	.0093
	61	Dough	.542	.98	6.84	1.44	.134	.0136
	68	Mature	.702	.88	9.06	2.47	.210	.0255
Sunrise kafir	55	Milk	.542	1.67	5.52	.91	.136	.0138
	62	Dough	.605	1.20	6.34	1.51	.190	.0164
	68	Mature	.725	.91	8.46	2.43	.250	.0255
Reeds kafir	56	Milk	.485	1.32	5.19	.90	.083	.0106
	63	Dough	.642	1.21	6.68	1.89	.135	.0180
	70	Mature	.685	1.09	7.82	2.11	.159	.0228

It will be observed from Table 8 that the percentage of ash in the dry matter decreased as maturity approached in all seven samples of the grain sorghum seeds examined. These results are in agreement with those obtained by André (2) while working with other types of seeds. Such results would naturally be expected, since the later stages of maturation of seeds is accompanied by the storing up of starches and fats and other organic and ash constituents. The per-

centage of protein ( $N \times 6.25$ ) in the dry material shows only a very small but definite increase, indicating that the rate of synthesis of this substance follows a course almost parallel to the increase in dry matter.

There was a definite increase in the percentage of fat in the dry matter of all seven varieties as maturity approached, thus indicating a storage of these substances during the later stages of development.

While the percentage of phosphorus in the dry seeds varied considerably with the different varieties, the amount of this substance in each variety showed a definite increase as maturity approached. The fact that the percentage of ash in these seeds decreases at the same time that the phosphorus increases indicates that this substance is entering some vital combination with the organic constituents of these tissues. With a few exceptions this increase in total phosphorus appeared to run parallel with the increase in fat content.

There is a substantial increase in the percentage of lipid phosphorus in every variety examined. While this increase is very significant, it is not sufficient to explain the total increase in phosphorus. Nor is it to be expected that it would, as Zaleski (34) has shown that there is an increase in protein phosphorus during the last stages of maturity. This increase in the percentage of lipid phosphorus is slightly greater than the increase in the percentage of either fat or total phosphorus, thus indicating an exceedingly rapid synthesis of the phospholipins in the seeds during the later stages of maturity.

An examination of Table 9, which contains the percentage of these substances in the fresh seeds, shows these increases to be much more pronounced. When considering the percentage of ash in the fresh seeds, a slight decrease in five of the seven varieties is found, while the other two remained almost constant. Such findings demonstrate that these seeds acquire their maximum ash content before or by the time they reach the milk stage. This change is followed by a slight decrease in these inorganic constituents as maturity approaches.

From the same table it is noted that the percentage of protein shows a definite increase in all cases. This increase appears to run almost parallel with the increase in dry matter, thus showing that the nitrogenous materials continue to be formed or stored in these seeds until maturity is reached. The percentage of fat, of total phosphorus, and of lipid phosphorus, each showed an even greater increase than did the protein. Such findings again would indicate a storage of these substances in the mature seeds. From the limited data obtained, no definite relationship could be established between the rates of formation or depositing of these three substances in the seeds other than the fact that all three appear to accumulate in the seeds simultaneously.

**EFFECT OF GERMINATION ON ASH, PROTEIN, FAT, TOTAL PHOSPHORUS, WATER SOLUBLE PHOSPHORUS, AND PHOSPHOLIPIN CONTENT OF SEEDS OF GRAIN SORGHUMS**

The effect of germination upon the composition of various seeds has been observed by a number of investigators, but the results have not always been in agreement. In general, the amounts of phospholipins in various seeds, at different stages of germination, appears to be influenced by the condition under which germination takes place.

Schulze and Steiger (28) found the phospholipins to decrease when the seeds were germinated in the dark, while Maxwell (21) observed a definite increase in phospholipins when the seeds were germinated in the light. Similar observations were made by Zaleski (32), Iwanoff (16), Bernardini and Chiarulli (6), and others. But Frankfurt (11) obtained contradictory results when he investigated the effect of germination upon the seeds of *Helianthus annuus*, and found an increase in the phospholipins when the seeds were germinated in the dark.

Deleano (10) observed the fat content to remain constant during the first eight days of germination and then decrease very rapidly. Bernardini and Chiarulli (6) germinated the seeds of some of the cereal grains and found that both the free and the combined phospholipins increased at about the same rate until the period of chlorophyll production, after which they decreased. In the absence of light, germination was accompanied by a loss of phospholipins, chiefly of the combined form. Hart and Andrews (13) found germinated seeds to be rich in soluble organic phosphorus, and drew the conclusion that the organic phosphorus of each—oats, maize, and wheat—is not transformed into inorganic phosphorus during germination. Zaleski's (33) experiments with the seedlings of *Lupinus agustifolius* indicate that the protein-containing phosphorus and the phospholipins (chiefly lecithin) are decomposed by enzymes during germination with the production of inorganic phosphates.

Zlataroff (35) made an analysis of the seeds of *Cicer aritum*, after 25 days of germination and found that the protein had decreased to about one-third of its original value, but that the nuclein content remained constant. Further observations by this investigator showed that the inorganic phosphorus increased at the expense of the protein and other more soluble phosphorus-containing compounds. The phospholipins did not decrease to the same extent, but appeared to act as a reserve substance. Van Laer and Duvinage (19) found the total phosphorus content of barley to decrease on germination, and a definite shift of phosphorus from the husk and endosperm to the nuclei and rootlets. These authors concluded that the phosphorus change from the endosperm to the nuclei was by way of the soluble phosphates.

From the results obtained in previous investigations concerning the effect of germination upon the composition of a number of different seeds, one is led to believe that the changes in composition during germination vary with the type of seeds under investigation, and more particularly, with the conditions under which germination takes place. With these facts in mind, an investigation was started to ascertain the effect of germination upon the composition of the seeds of the grain sorghums.

The seeds of two common varieties of grain sorghum, differing in composition as widely as possible, were selected as experimental materials. These were darso and yellow milo. The experiment was so planned as to note the effect of sunlight and darkness on the germination of seeds of both varieties.

About 5 kgm. of seeds from each variety were washed free of dust, dirt, and hulls, and carefully rinsed three times with distilled water. They were then spread upon a screen and allowed to dry for two days at room temperature. At the end of that time about  $2\frac{1}{2}$  kgm. of the plumpest seeds were hand-picked from each variety. Twenty-

two samples of 100 gm. each from each variety were weighed to the nearest tenth of a gram, and preserved in small glass containers with screw tops. The germination was carried on in small enamel pans, about 8 inches in diameter. A perforated desiccator plate was placed in the bottom of each pan, and a piece of hardened filter paper placed upon this plate. The 100 gm. sample of seeds was spread uniformly upon the filter paper, where they were kept moist by distilled water from the bottom of the pan, by capillarity.

At the beginning of the germinating procedure, two of the 100 gm. samples from each variety were combined and reserved as the ungerminated sample. Ten of the remaining samples were put in the germinating pans, five pans being placed in a south window and five in a dark cabinet. The temperature of the room was maintained at about 32° C. by means of an electric hot plate. The period of germination varied from 2 to 10 days, the seeds being kept moist at all times by the addition of distilled water to the bottom of the pan by means of a pipette. At the end of the period of germination, the seeds were carefully raked from the filter paper into the bottom of the pan, the filter paper being pressed in order to free it of as much dissolved material as possible. The paper was then washed with a small amount of distilled water and again pressed. This process of washing the paper was repeated twice more, a small volume of water being used each time. The perforated desiccator plate was rinsed with water and removed from the pan. This precaution was taken to prevent any unnecessary loss of soluble constituents. The pan containing the seeds and the washings was maintained at a temperature of about 50° until dry. This usually required about four hours. When dry, the total contents of the pan were removed by means of a spatula and a camel's-hair brush, and placed in a glass container. This process was continued until five samples from each variety had been germinated in both light and darkness, for intervals varying from 2 to 10 days.

Since there were two cloudy days during this series of germinations, which undoubtedly would have some effect upon the experiment, and since a larger number of germinated samples would tend to decrease any error that might have been made in the germination of a particular sample, the other 10 samples from each variety were germinated in a similar manner. This time there were three and one-half cloudy days during the period of germination.

When the process of germinating the 40 samples had been completed, each of the 2 samples germinated for the same length of time and under similar conditions were combined, thus forming a total of 22 samples, including 20 germinated samples and 2 ungerminated. The seeds were then dried for four hours at 100° C. and weighed. The difference between the loss in weight of the germinated samples and the ungerminated sample of the two respective varieties, represented the loss in dry matter during the germinative process.

The samples were then ground until the total mass passed through a 60-mesh sieve, and preserved in air-tight containers until the desired analysis could be made.

The methods of analysis were the same as those employed in the earlier experiments, except the estimation of the water-soluble phosphorus, which has not previously been mentioned.

The water-soluble phosphorus determination was carried out in the following manner: Ten grams of the dry ground seeds were placed upon a small filter paper folded to fit a small funnel, and eight 25 c. c. portions of hot water were poured upon this material in succession at such a rate that each portion had passed through the filter before the succeeding portion was added. The total extract, which measured slightly less than 200 c. c., owing to the small quantity retained by the sample, was collected in 500 c. c. Erlenmeyer flasks. The flasks were immediately corked tightly and immersed in a cold-water bath to reduce the chances of hydrolysis to a minimum. When cold, 10 gm. of trichloroacetic acid was added to each sample of extract, the flasks shaken, and returned to the cold-water bath, where they remained for four hours, being shaken occasionally during this period. At the end of this time, when the proteins had been precipitated, the extracts were filtered through dry quantitative filter paper and collected in a dry 400 c. c. beaker. One hundred cubic centimeters of this filtrate was placed in a 200 c. c. porcelain evaporating dish and evaporated to dryness on a water bath. The residue in the evaporating dish was then moistened with 10 c. c. of 10 per cent solution of sodium carbonate and again evaporated to dryness. The evaporating dish was placed in an electric muffle and the contents ignited to dull redness. When the evaporating dish had cooled, the contents were dissolved in a small quantity of warm dilute nitric acid, and transferred to a 400 c. c. beaker by rinsing with distilled water. The total phosphorus content of this solution was determined by the usual gravimetric method. The results are expressed as the percentage of soluble phosphorus in the 10 gm. of sample. This process was repeated until at least two consistent values had been obtained for each sample.

The writer is aware that such a procedure is open to some criticism, but even at that, eliminating experimental errors, such a procedure should at least give comparative values for the changes during germination. This is all that can be hoped for until more is known concerning the various changes which take place during germination and more accurate means are devised to measure them.

In this investigation all determinations were made in duplicate and repeated until consistent checks were obtained.

For ease of study the results are shown in table form. (See Tables 10 and 11.)

After two days of germination the seeds appeared very much enlarged but no sprouts were visible. After four days, about 70 per cent had sprouted or showed evidence of sprouting. After six days, about 90 to 95 per cent had sprouts averaging about one-half inch in length. At this time the seeds grown in the light were larger and had a much better color than those grown in the dark. The difference in the appearance of the two groups was yet more evident at the end of the 8-day period. At the end of 10 days the sprouts from the seeds germinated in the light had reached the height of about  $1\frac{1}{2}$  inches and had begun to show evidence of chlorophyll formation, while the sprouts from the seeds germinated in the dark were much more irregular in height, showed no evidence of chlorophyll production, and all of the sprouts had developed a pale yellowish color.

TABLE 10.—Percentage of ash, protein, fat, total phosphorus, water-soluble phosphorus, and lipid phosphorus in yellow milo and darso seeds germinated in the light and in the dark, determined on a dry-matter basis

Seeds used	Condition under which germination occurred	Sample	Days of germination	Dry matter	Ash	Protein	Fat	Total phosphorus	Water-soluble phosphorus	Lipid phosphorus
				<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Yellow milo.....	Light.....	A.....	0	93.10	1.89	11.04	3.18	0.242	0.055	0.0451
		B.....	2	92.00	1.92	10.62	3.10	.248	.073	.0476
		D.....	4	90.10	1.92	9.85	2.92	.257	.081	.0572
		F.....	6	88.20	1.95	9.44	2.65	.254	.098	.0645
		H.....	8	86.40	1.96	9.03	2.44	.274	.105	.0684
		J.....	10	83.00	1.99	8.60	2.08	.282	.118	.0870
	Dark.....	C.....	2	89.20	1.90	10.37	2.96	.247	.071	.0471
		E.....	4	87.40	1.94	9.75	2.78	.251	.083	.0491
		G.....	6	86.50	1.96	8.97	2.57	.258	.096	.0516
		I.....	8	85.10	1.98	8.85	2.41	.254	.113	.0540
		K.....	10	84.00	1.98	8.74	2.18	.275	.126	.0624
		L.....	0	93.50	1.42	12.69	4.05	.235	.062	.0364
Darso.....	Light.....	R.....	2	92.10	1.46	12.31	3.91	.237	.069	.0384
		S.....	4	89.30	1.48	12.12	3.85	.240	.083	.0406
		T.....	6	84.00	1.54	12.10	3.71	.250	.105	.0442
		U.....	8	82.20	1.56	11.41	3.56	.255	.124	.0462
		V.....	10	81.90	1.59	11.34	3.32	.262	.138	.0503
		M.....	2	92.00	1.44	12.09	3.78	.242	.060	.0386
	Dark.....	N.....	4	88.10	1.48	11.16	3.58	.251	.081	.0410
		O.....	6	84.30	1.51	11.12	3.30	.261	.090	.0419
		P.....	8	82.50	1.57	11.06	3.03	.267	.119	.0428
		Q.....	10	82.00	1.58	11.06	3.00	.269	.134	.0442

TABLE 11.—Percentage of ash, protein, fat, total phosphorus, water-soluble phosphorus, and lipid phosphorus in yellow milo and darso seeds germinated in the light and in the dark, determined on the fresh material

Seeds used	Condition under which germination occurred	Sample	Days of germination	Factor	Ash	Protein	Fat	Total phosphorus	Water-soluble phosphorus	Lipid phosphorus
					<i>P. ct.</i>	<i>Per ct.</i>	<i>Per ct.</i>	<i>Per ct.</i>	<i>Per cent</i>	<i>Per cent</i>
Yellow milo.....	Light.....	A.....	0	1.0000	1.89	11.04	3.18	0.242	0.055	0.0451
		B.....	2	.9892	1.90	10.52	3.07	.245	.072	.0471
		D.....	4	.9677	1.86	9.44	2.86	.248	.079	.0544
		F.....	6	.9462	1.85	8.94	2.54	.241	.093	.0610
		H.....	8	.9236	1.81	8.34	2.25	.253	.097	.0632
		J.....	10	.8923	1.78	7.67	1.86	.252	.105	.0776
	Dark.....	C.....	2	.9569	1.82	9.92	2.83	.237	.068	.0451
		E.....	4	.9344	1.81	9.11	2.60	.234	.077	.0459
		G.....	6	.9236	1.81	8.28	2.37	.238	.089	.0477
		I.....	8	.9139	1.81	8.10	2.20	.241	.103	.0494
		K.....	10	.9032	1.79	7.89	1.97	.249	.114	.0564
		L.....	0	1.0000	1.42	12.69	4.05	.235	.062	.0364
Darso.....	Light.....	R.....	2	.9839	1.43	12.11	3.85	.238	.068	.0378
		S.....	4	.9518	1.41	11.54	3.69	.228	.079	.0386
		T.....	6	.8983	1.38	10.87	3.33	.224	.094	.0397
		U.....	8	.8780	1.37	10.02	3.13	.224	.110	.0406
		V.....	10	.8780	1.40	9.96	2.91	.230	.121	.0442
		M.....	2	.9840	1.42	11.90	3.72	.238	.059	.0380
	Dark.....	N.....	4	.9412	1.39	10.51	3.37	.237	.076	.0386
		O.....	6	.8980	1.36	9.99	2.96	.234	.080	.0385
		P.....	8	.8823	1.39	9.76	2.67	.236	.105	.0378
		Q.....	10	.8750	1.38	9.68	2.63	.235	.117	.0387

When the percentages of the various constituents in the dry material are considered, a number of changes are apparent. (Table 10.) Most conspicuous of these is the marked decrease in dry matter as the periods of germination were increased. Corresponding decreases

were observed with the two varieties of seeds under similar conditions, but the decreases were slightly less marked when germination took place in the dark. However, the rate of decrease was greater during the first few days of germination in the dark than during a similar period of germination in the light. Since precautions were taken to prevent possible losses of the soluble constituents, the loss in dry matter must be attributed to the germinative processes going on in the seeds during the germination period.

There appears to be a very slight increase in the percentage of ash in the dry matter. (Table 10.) This increase is almost proportional to the decrease in dry matter. Such a relationship is exactly what is to be expected if there had been no loss of inorganic materials by seepage.

The percentage of both the protein and the fat decreased very rapidly during the 10 days of germination. Similar observations have been made by investigators working with other seeds. There appears to be some relationship between the rate of decrease of the protein and the fat of these seeds during germination but the ratio is not definite.

The percentage of total phosphorus ran parallel with the ash, and increased, with a decrease in the percentage of dry matter. This again is to be expected as there was little chance for a loss of this substance.

The water-soluble phosphorus and the lipid phosphorus both made an enormous increase during the 10-day period. During this time the percentages of these substances increased from approximately 20 to 130. The increase in the water-soluble phosphorus must have resulted from the splitting off of this element from some of its conjugated products, possibly the proteins. The increase in the percentage of this fraction was proportional to the increase in the percentage of lipid phosphorus. Such findings would suggest that during germination the phosphorus in the seed passes through the water-soluble stage before forming the more vital plant tissue by combining with the lipids.

Table 11 contains the same data as Table 10 but expressed as percentage of the original seed. These data bring out a few facts which are not so evident from Table 10. When the percentage of ash in the original material is considered, it is found to be constant throughout the several periods of germination. Slight variations are evident but these are within the limits of experimental error. This also holds true for the percentage of total phosphorus.

Experiments were conducted to ascertain the relative amounts of phospholipins in various seeds and to determine the relation of the phospholipins to the ash, protein, fat, and total phosphorus present in the seeds.

The phospholipin content of seeds was found to be as variable as many of the other constituents. The greatest variation in the amounts of these substances occurs between different types of seeds, but minor variations are apparent between different varieties of the same type or even different samples of the same variety. The phospholipin content is independent of the color and size of the seeds. In general, the highest percentages of phospholipins are found in the seeds having the highest protein and the highest fat content, but this is not always true. The total phosphorus increases with increase

of phospholipins but the relationship is not definite. Some of these irregularities could no doubt be eliminated by taking into consideration the conditions of the soil on which the seeds were grown, together with the climatic conditions during the period of growth and maturity.

The ash, protein, fat, total phosphorus, and phospholipin content of seven varieties of grain-sorghum seeds, at three different stages of growth were determined. The results obtained demonstrate very clearly a definite increase in all of the constituents, with the exception of ash, as the seeds approached maturity. The number of samples examined does not permit a definite correlation between the rate of formation of any two of these substances.

Two samples of grain sorghum seed were germinated both in light and in darkness and the percentage of several of their constituents determined at various periods of germination. It was found that the seeds lost about 10 per cent of dry-matter during 10 days of germination. This loss appeared to be slightly less when germination took place in the dark. The percentage of ash in the dry-matter increased, but the total amount of ash remained constant. The percentage and amount of both protein and fat decreased in the dry-matter in all cases. The percentage of total phosphorus paralleled that of the ash. Both the percentage and amount of water-soluble phosphorus and lipid phosphorus increased. The increase in phospholipins, estimated as lipid phosphorus, was almost directly proportional to the increase in water-soluble phosphorus. The lipid phosphorus showed a greater increase when germination took place in the light. There appears to be no relationship between the increase in lipid phosphorus and the changes in ash, protein, fat, or total phosphorus.

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# RELATION OF TEMPERATURE TO RATE AND TYPE OF FERMENTATION AND TO QUALITY OF COMMERCIAL SAUERKRAUT<sup>1</sup>

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## INTRODUCTION

The fermentation of cabbage by lactic acid-forming microorganisms is greatly influenced by several factors. Among these factors temperature is undoubtedly one of the most important. It controls the rate and kind of fermentation and thus has a marked effect on the quality of the product.

Sauerkraut packers seldom attempt to control the initial temperature of the shredded cabbage, with the result that the temperature of the different vats varies widely at different times during the packing season. During the latter part of August cabbage is often packed at 80° F. or higher, which is the temperature suggested by LeFevre (2)<sup>3</sup> for making sauerkraut. Wehmer (6), on the other hand, suggests that 40° is the proper temperature. This low temperature is not uncommon for cabbage shredded in November and December.

The initial temperature of the shredded cabbage largely controls the temperature of the fermentation. The temperature of the factory during fermentation has only a slight effect on the temperature of the cabbage, for the regular-size vats are large and contain 40 to 50 tons of shredded cabbage. On the other hand, it is conceivable that room temperature may have an effect on the temperature of small quantities of cabbage. Room temperature undoubtedly had an effect in the case of the top and bottom portions of the experimental vats used in this study, which contained only 8 tons of cabbage.

## EXPERIMENTAL METHODS

The experiments described in this paper were arranged to study the relation of temperature to rate and type of fermentation and to the quality of sauerkraut made under factory conditions. They were conducted in a commercial sauerkraut factory near Milwaukee, Wis.

Great care was exercised to secure uniformity of material and conditions during the experiments, especially in the filling of the vats. The cabbage used was from one field, was of the same age and variety, and had been stored for about two weeks in outside ventilated piles, 90 feet long, 10 feet wide, and 6 feet high. The piles were opened along one side for the entire length, and each load

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<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 1038.

removed was representative of the whole pile. The temperature of this cabbage after being cored and cut was about 40° F. It was warmed to the desired temperatures by means of steam jets placed along a chute through which the shredded cabbage fell.

Eight vats of 8-ton capacity each were filled in two series. These oval vats were 7 feet deep. The short diameter was about 9 feet and the long diameter 11 feet. Four vats were used in the first series and an equal number in the second. Their temperatures were approximately 45°, 55°, 65°, and 75° F., respectively, when filled. The second series of vats was packed four days after the first, and was as nearly as possible a duplicate of the first series. Two days were required to fill the four vats in each series.

Dividing the eight vats into two series proved another safeguard in avoiding variation in cabbage. The four vats of each series were packed simultaneously by alternating the cartloads of cut cabbage containing about 700 pounds each. This procedure made it certain that each vat contained cabbage similar to that in the others.

Three thermocouples were put into each vat, one 2 feet from the bottom and an equal distance from the side, another in the center of the vat, and another 2 feet from the top and an equal distance from the side opposite the bottom thermocouple. These thermocouples were carefully checked before being used, and readings obtained with them while in the vats were verified by means of thermometers.

Two thick-walled rubber tubes with small Büchner funnels on the ends were placed in each vat, so that a sample of juice could be drawn from the center and bottom when desired. For the first 10 days samples were drawn at 24-hour intervals, and later at more irregular intervals. From these samples the following data on bacteria, acidity, and temperature were obtained.

The number and activity of the bacteria were measured in three ways: (1) By direct counts (Breed method); (2) by determining the time of reduction of methylene blue; (3) by the acid production in the fresh cabbage juice.

Titrateable acidity was measured by boiling 10 c. c. of the samples for a few seconds to expel CO<sub>2</sub>, and then titrating with 0.1 N NaOH, phenolphthalein being used as an indicator. The hydrogen-ion concentration of each sample was determined by the colorimetric method.

Changes of temperature in different parts of the vats during fermentation were observed by means of the thermocouples.

## METHODS OF ANALYSIS

### CABBAGE

Representative heads were selected for chemical analysis from the inside and outside of the pile in which the cabbage was stored. These were shredded, thoroughly mixed, 20 gm. of the material were dried for 24 hours in an oven at about 110° C., and the loss in weight calculated as moisture. Total nitrogen was determined in a 10 gm. sample of cabbage by the Kjeldahl method. To determine the water-soluble constituents, 400 gm. of cabbage were thoroughly ground, filtered, and washed to a total volume of 2 liters. Nitrogen was determined in this extract by the Kjeldahl method, amino acids by

the Van Slyke (5) procedure, and reducing sugars by the Shaffer-Hartmann (3, 4) method. Sucrose was determined by the acid-hydrolysis procedure. After the fermentation in each vat was completed, a composite sample was taken and the products of fermentation determined.

Table 1 shows the composition of cabbage from different parts of the piles used in this experiment.

#### SAUERKRAUT

One hundred cubic centimeters of sauerkraut juice were subjected to steam distillation after 5 c. c. of 1 N  $H_2SO_4$  had been added. The volatile acid obtained was titrated with 0.1 N NaOH. The non-volatile acid in the residue from the steam distillation was separated and the quantity determined by the Kutscher-Steudel (1) method. The alcohol in 100 c. c. of sauerkraut juice was determined by neutralizing the volatile acids present and distilling off the alcohol. The latter was then oxidized with potassium dichromate to acetic acid, which was distilled off and titrated with 0.1 N NaOH. The reducing sugars present in the sauerkraut juice were determined by the Shaffer and Hartmann method.

TABLE 1.—Composition of cabbage from different parts of piles used in experiment

Item	Wet basis		Dry basis	
	Outside	Center	Outside	Center
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Moisture.....	92.300	90.700	0.0	0.0
Total nitrogen.....	.225	.242	2.93	2.60
Water-soluble nitrogen.....	.107	.120	1.39	1.29
Amino nitrogen.....	.063	.076	.82	.82
Reducing sugar.....	2.660	2.596	34.51	27.83
Sucrose.....	.717	.808	9.32	8.64

#### COMPOSITION OF CABBAGE

Table 1 indicates the necessity of thoroughly mixing the cabbage as was done in the eight experimental vats, in order to obtain a uniformity of raw material. The manner in which this was accomplished has been previously described. Apparently variations in composition exist between heads selected from different parts of the pile in which they were stored. The greatest difference is in the sugar content. The cabbage at the center of the pile had lost sugar through excessive respiration. These heads were much warmer than those on the outside of the pile, which indicates a more rapid rate of respiration.

#### CHANGES IN TEMPERATURE DURING FERMENTATION OF CABBAGE

Figure 1 shows the changes in temperature at the center of vats 1, 3, 4, and 6 at successive intervals during fermentation. Changes in the factory temperature from day to day are also shown. It is obvious that there is a rise in temperature during the first part of the

fermentation which can not be attributed to conduction from the room, since the latter is at a lower temperature than the vats. It is difficult to tell whether the heat is supplied by bacterial or plant-cell activity. During the latter period of the fermentation the temperature of the vats tends to adjust itself to room temperature. In most cases, the rise in temperature occurs when acidity is increasing most rapidly, and falls off after this rapid increase ceases. The temperature curves of Figures 2, 8, 9, and 10 illustrate this point. This rise in temperature affords some evidence that heat is supplied during the fermentation by bacterial action rather than by plant cells alone. Furthermore, the initial rise in temperature often extends over a period of eleven or twelve days. It seems very doubtful if the activity of plant cells alone could account for a rise in temperature over as long a period of time. It is interesting to note that the rise

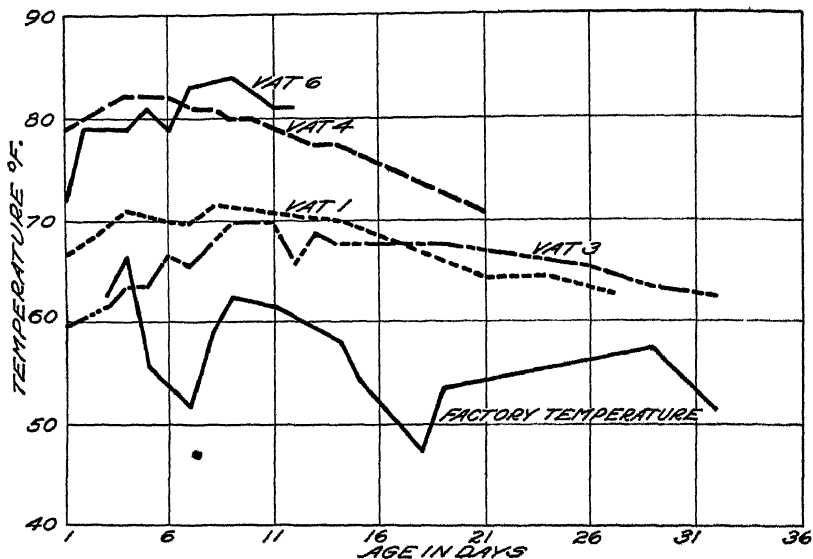


FIG. 1.—Temperature at the center of the warm vats at various times during fermentation

in temperature in all eight vats was about 10° F., and that the rate at which this rise in temperature occurred, in general, parallels the rate of fermentation (acid production).

In Table 2 are recorded the changes in temperature occurring in the three different parts of the experimental vats. It appears that the temperature at the top and bottom of each vat is more quickly affected by changes in factory temperature than that at the center. Thus the temperature at the top of vat 2, filled at 44° F., was higher than that at the center and more nearly adjusted itself to factory temperature than did the latter. In the case of the warm vat, 6, the bottom and top temperatures were the same as the center temperature at the beginning of the fermentation, but dropped off more quickly and were nearer the temperature of the factory than the latter. It should also be noted that in general the temperature at the bottom of each vat was lower than at the top. It is rather

difficult to explain this fact unless possibly it was due to convection currents carrying the heat liberated during fermentation to the surface.

TABLE 2.—Changes in temperature of sauerkraut during fermentation

[T=top sample, C=center sample; B=bottom sample]

Age of sauer- kraut (days)	Tem- per- ature of factory (°F.)	Temperatures (° F.) in—															
		Vat 2 (initial temperature 44°)			Vat 9 (initial tem- perature 50°)	Vat 7 (initial tem- perature 52°)	Vat 5 (initial temperature 53°)			Vat 3 (initial temperature 64°)			Vat 1 (initial tem- perature 65°)	Vat 6 (initial temperature 74°)			Vat 4 (initial tem- perature 76°)
		T	C	B	C	C	T	C	B	T	C	B	C	T	C	B	C
1		41	46	44	51	50	52	55	56	61	60	66	67	77	71	75	79
2		44	45	44	51	55	55	56	58	63	61	66	68	79	79	77	80
3	63	46	44	44		56				62	62	66		79	79	79	
4	67	48	44	44	54	55	60	60	60	66	64	66	71	79	79	78	82
5	56	48	43	44		55				66	64	66		82	81		
6	54	52	45	47	55	56	62	60	60	67	67	67	70	79	79	78	82
7	52	52	45	48	55	57	63	61	59	67	66	66	70	81	83	76	81
8	59				56		63	61	61				72				81
9	63	55	47	50	57	57	62	61	60	70	70	66	72	79	84	75	80
10					57		62	61	60				71				80
11	62	56	47	50		58				67	70	65		77	81	72	
12		56	48	50		59				66		63		77	81	72	
13	60	60	50	52	58	59	63	63	61	68	69	65	71				77
14	59	60	50	52	58	60	62	64	60	67	68	63	71				77
15	55	60	51	53		60				67	68	63					
18	48	62	55	55		61				67	68	63					
19	54	60	54	53		63				66	68	62					
21					61		62	61	58				65				71
24					63		61	62	58				65				
26		61	59	55		62				63	66	60	64				
29	57	61	58	55		63				62	64	59					
32	52	60	58	55		62				61	63	58					
41	53	55	57	51	59		55	57	52								

## TITRATABLE ACID AND HYDROGEN-ION CONCENTRATION

Figures 2 and 3 illustrate clearly the relationship between titratable acidity and hydrogen-ion concentration, and also the general correlation which exists between samples taken from different parts of a single vat. The changes in titratable acidity at the center and bottom of each vat are shown in Table 3. It appears from this table that the rate of acid formation is largely dependent on the temperature. It can be seen that it was necessary for fermentation to go on for 11 days in the vat whose initial temperature was 44° F. before an acidity of 0.5 per cent was reached. This period was shortened to six days in the vat whose initial temperature was 53° F., to 3 days in the 65° vat, and to 24 hours in the 76° vat. When the temperature of the shredded cabbage is between 65° and 75° the acidity increases rapidly during the first part of the fermentation, after which it increases slowly. When the temperature is below 65° the rise in acidity is more gradual and uniform throughout the entire process. These facts are clearly brought out in Figures 4 to 10, inclusive, in which changes in acidity at the center of each vat are shown. It is apparent from the data presented in Table 3 that the acidity at the bottom of each vat is about the same as at the center.

In Table 4 are recorded the results of the  $P_H$  determinations. It is apparent that these are of little value as measures of the degree of

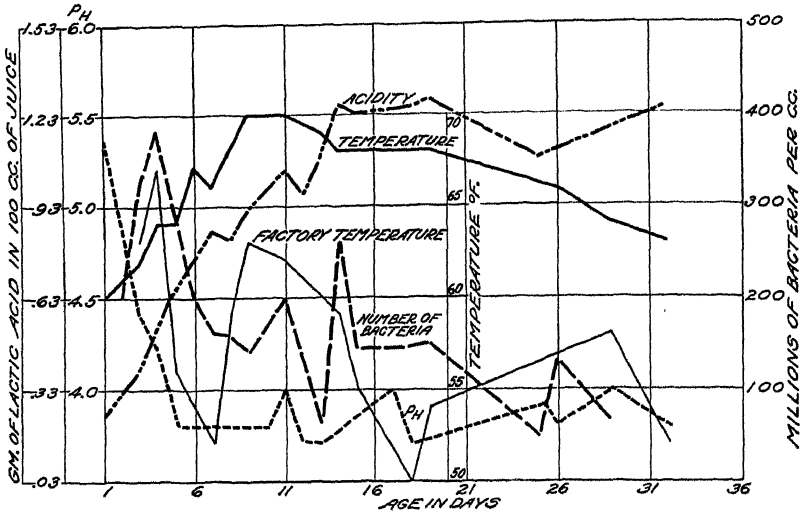


FIG. 2.—Titratable acidity, hydrogen-ion concentration, number of bacteria, and temperature at the center of vat 3 at various times during fermentation

fermentation, since in every case the  $P_H$  drops more or less rapidly, depending upon the temperature of the fermentation, to about 3.7,

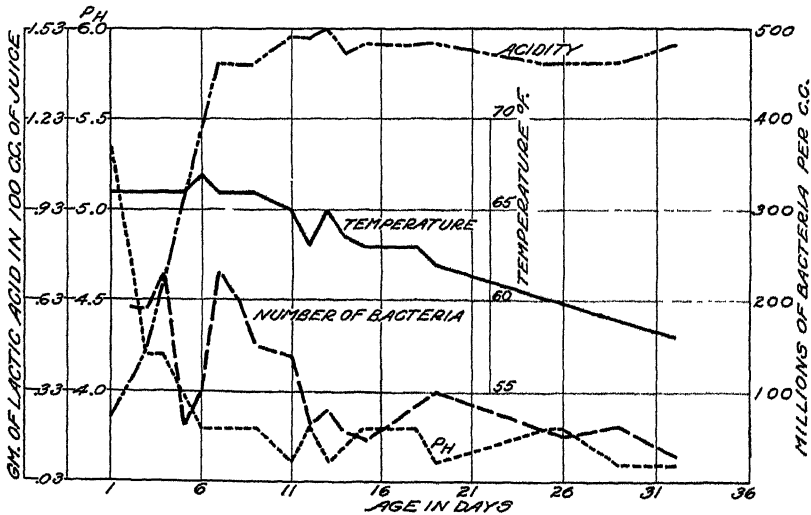


FIG. 3.—Titratable acidity, hydrogen-ion concentration, number of bacteria, and temperature at the bottom of vat 3 at various times during fermentation

and does not go any lower than this even though the titratable acidity continues to rise.

TABLE 3.—*Changes in titratable acidity of sauerkraut during fermentation*

[C=center sample; B=bottom sample]

Age of sauerkraut (days)	Percentages of lactic acid in—															
	Vat 2 (initial temperature 44° F.)		Vat 9 (initial temperature 50° F.)		Vat 7 (initial temperature 52° F.)		Vat 5 (initial temperature 53° F.)		Vat 3 (initial temperature 64° F.)		Vat 1 (initial temperature 65° F.)		Vat 6 (initial temperature 74° F.)		Vat 4 (initial temperature 76° F.)	
	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B
1	0.17	0.14	0.09	0.09	0.15	0.14	0.09	0.16	0.18	0.23	0.37	0.19	0.38	0.52	0.59	0.64
2			.28	.23			.30	.37			.46	.26			.83	1.01
3	.15	.08	.28	.25	.39	.27	.38	.49	.40	.47	.57	.40	.87	1.06	.80	1.02
4	.18	.10	.32	.28	.61	.28	.46	.70	.54	.72	.62	.41	1.05	1.14	1.04	1.21
5	.25	.14			.64	.47			.66	.98			1.30	1.27		
6	.28	.19	.37	.46	.74	.50	.55	.82	.74	1.21	.85	.65	1.23	1.39	1.21	1.27
7	.36	.21	.42	.55	.71	.70	.53	.85	.85	1.39	.93	.70	1.24	1.63	1.31	1.32
8	.37	.26	.51	.71	.92	.74	.64	1.01	.82	1.38	1.14	.74	1.40	1.63	1.38	1.55
9	.39	.38	.58	.68	1.11	.85	.67	1.07	.92	1.39	1.16	.73	1.41	1.55	1.59	1.55
10			.67	.75			.81	1.09			1.33	.88			1.63	1.56
11	.55	.52			1.05	.95			1.05	1.51	1.35		1.36	1.63		
12	.60	.52			1.11	1.03			.97	1.50			1.15	1.64		
13	.70	.63	.84	.88	1.21	1.11	1.07	1.13	1.09	1.54	1.38	.87			1.68	1.68
14	.66	.68	.81	.85	1.09	1.13	1.13	1.13	1.26	1.45	1.53	1.04			1.72	1.57
15	.72	.64			1.06	1.21			1.23	1.48						
18	.75	.77			1.06	1.27			1.25	1.47						
19	.82	.76	1.13	1.05	1.30	1.29	1.31	1.33	1.28	1.48	1.56	.91			1.80	1.66
21			1.03	1.05			1.29	1.33			1.57	1.06			1.76	1.72
25	.91	.90	1.32	1.11	1.32	1.43	1.40	1.42	1.09	1.41						
27			1.41	1.24	1.48		1.46	1.42			1.49	1.20				
29	.97	1.06			1.31	1.46			1.19	1.42						
32	1.03	1.17			1.31	1.40			1.25	1.47						
41	1.31	1.59	1.64	1.17			1.66	1.62								

TABLE 4.—*Changes in the  $P_H$  values of sauerkraut during fermentation*

Age of sauerkraut (days)	PH values in center of—								
	Vat 2 (initial temperature 44° F.) <sup>a</sup>		Vat 9 (initial temperature 50° F.)	Vat 7 (initial temperature 52° F.)	Vat 5 (initial temperature 53° F.)	Vat 3 (initial temperature 64° F.)	Vat 1 (initial temperature 65° F.)	Vat 6 (initial temperature 74° F.)	Vat 4 (initial temperature 76° F.)
	C	B							
1	5.8	5.8	5.9	5.8	5.9	5.4	5.7	5.4	5.5
2			5.2		5.0		4.4		4.6
3	5.9	5.9	4.6	5.8	4.3	4.4	4.0	4.2	4.0
4	5.8	5.9	4.2	5.5	4.0	4.2	3.8	4.2	3.8
5	5.4	5.6		5.2		3.8		3.8	
6	5.3	5.4	4.2	4.5	4.0	3.8	4.0	3.8	3.8
7	5.0	4.8	3.9	4.4	4.0	3.8	3.8	3.8	3.7
8	4.6	4.4	4.0	4.2	3.8	3.8	3.7	3.6	3.5
9	4.2	4.2	4.2	4.0	3.8	3.8	3.6	3.6	3.5
10			4.0		3.9	3.8	3.6		3.5
11	4.2	4.2		4.0		4.0		4.0	
12	4.0	4.0		3.7		3.7		3.8	
13	4.0	4.1	3.9	3.9	3.8	3.7	3.6		3.6
14	4.2	4.2	3.9	3.9	3.8	3.8	3.7		3.6
15	4.2	4.2		3.9		3.9			
18	4.0	4.0		3.8		4.0			
19	4.2	4.0		3.8		3.7			
25	4.0	3.8	3.6	3.8	3.6	3.9	3.6		3.5
26	4.0	3.8		3.8		3.8			
29	3.9	3.8		3.8		4.0			
32	4.0	3.6		3.7		3.8			

<sup>a</sup> For comparison the  $P_H$  value of bottom sample is given

Additional evidence concerning this question was obtained by an analogous experiment in the laboratory. It was found that the  $P_H$  of fresh cabbage juice is 5.9. When the juice was saturated with

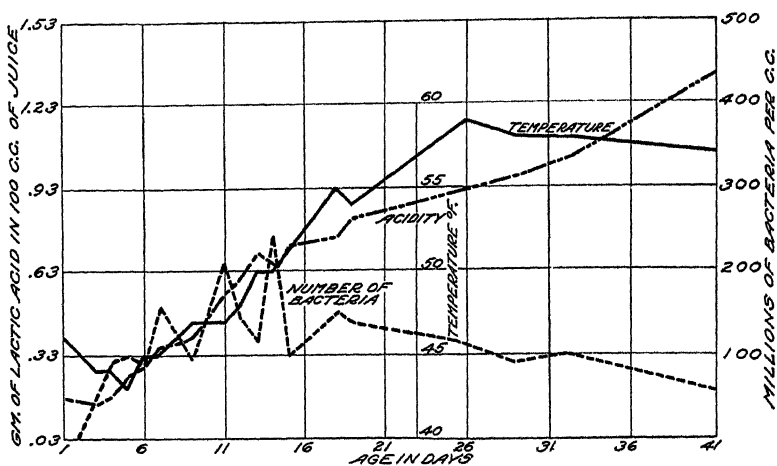


FIG. 4.—Temperature, acidity, and number of bacteria at the center of vat 2 at various times during fermentation

carbon dioxide, the  $P_H$  was lowered to 4.8. Large quantities of carbon dioxide are liberated during the first few days of fermentation of cabbage, which must almost completely saturate the juice ex-

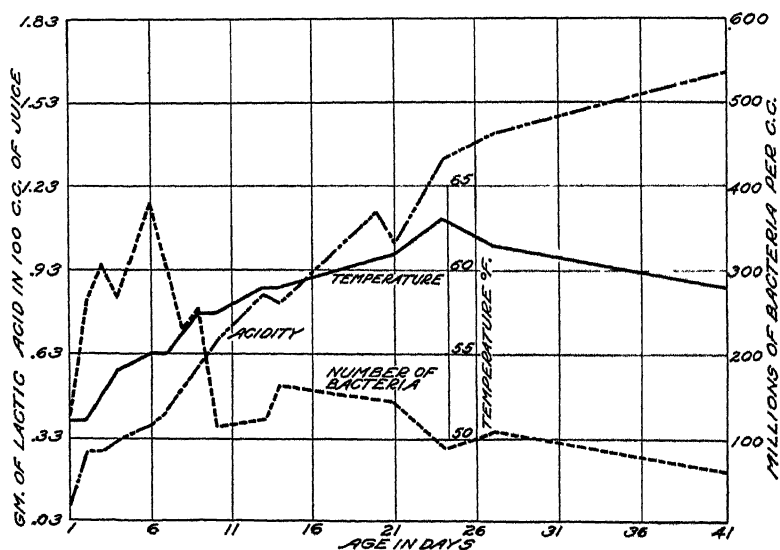


FIG. 5.—Temperature, acidity, and number of bacteria at the center of vat 9 at various times during fermentation

pressed from the latter. This would, in a large part, account for the rather sudden drop in  $P_H$  observed during the early days of fermentation before much acid had been formed.

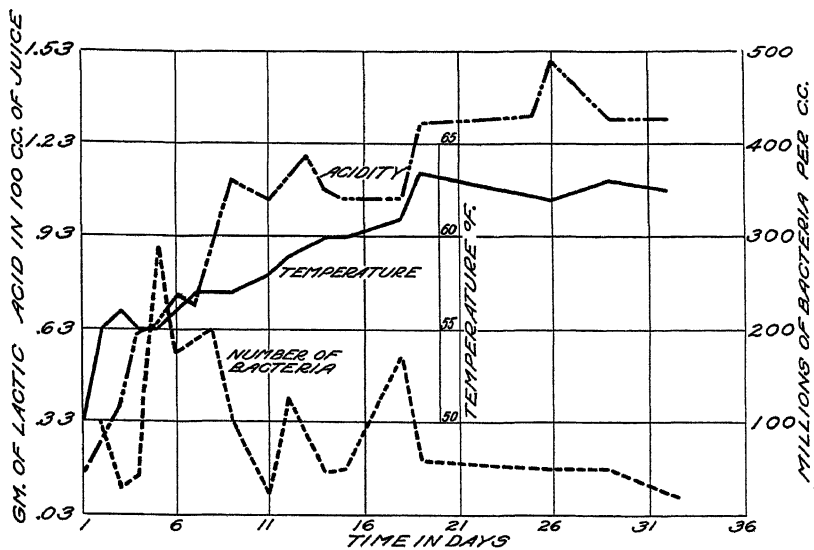


Fig. 6.—Temperature, acidity, and number of bacteria at the center of vat 7 at various times during fermentation

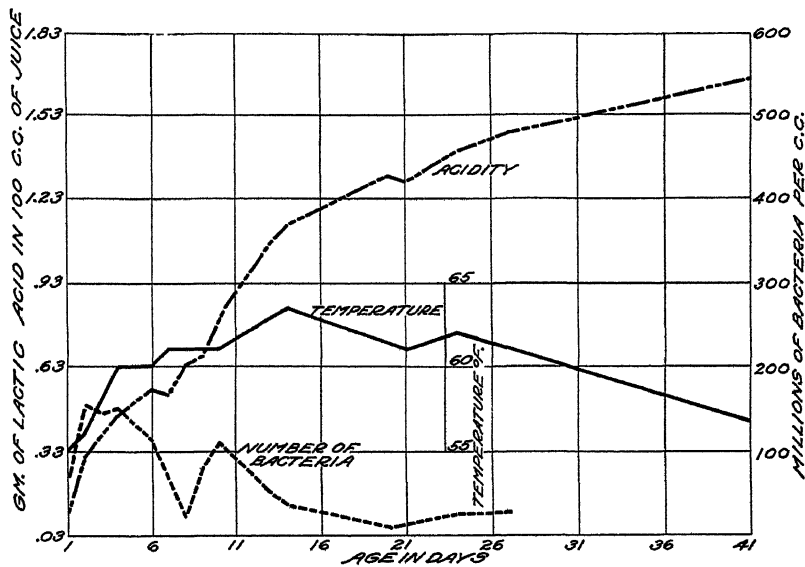


Fig. 7.—Temperature and acidity at the center of vat 5 and number of bacteria at the bottom of vat 5 at various times during fermentation

If 0.5 c. c. of 1 N. acetic acid and 1.3 c. c. of 1 N lactic acid are added to 15 c. c. of cabbage juice, a mixture is formed which has a  $P_H$  of 3.6 and contains quantities of the two acids equivalent to that

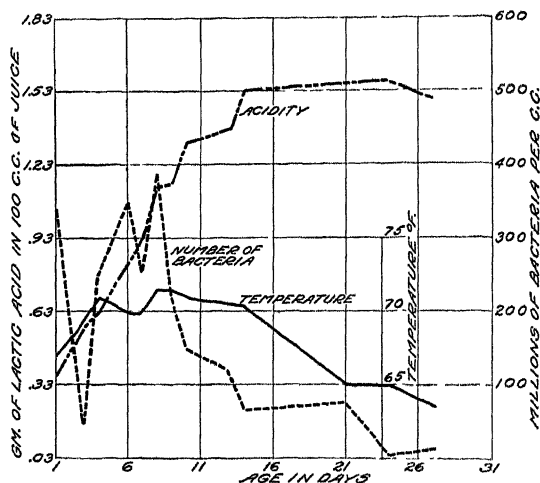


FIG. 8.—Temperature, acidity, and number of bacteria at the center of vat 1 at various times during fermentation

found in completely fermented cabbage. If the same quantities of these two acids are added to water, in which case no buffer effect is possible, the mixture will have a  $P_H$  of 2.9. These values indicate the buffering property of cabbage juice. If 0.25 c. c. of 1 N acetic

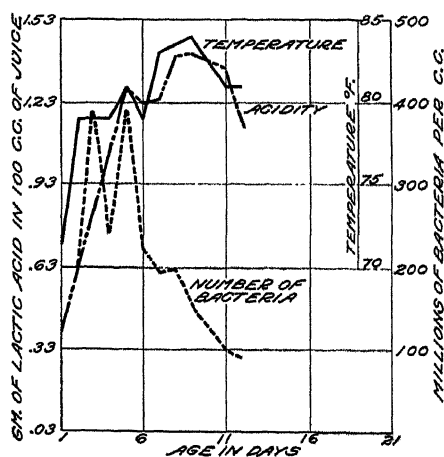


FIG. 9.—Temperature, acidity, and number of bacteria at the center of vat 6 at various times during fermentation

acid and 0.65 c. c. of 1 N lactic acid are added to 15 c. c. of cabbage juice, a mixture is formed having a  $P_H$  of 3.6 and containing quantities of the two acids equivalent to that found in half-fermented cabbage.

Since reducing the amount of acids one-half has no effect on the  $P_H$ , it is clear that  $P_H$  determinations are of but little value as a measure of changes in acidity.

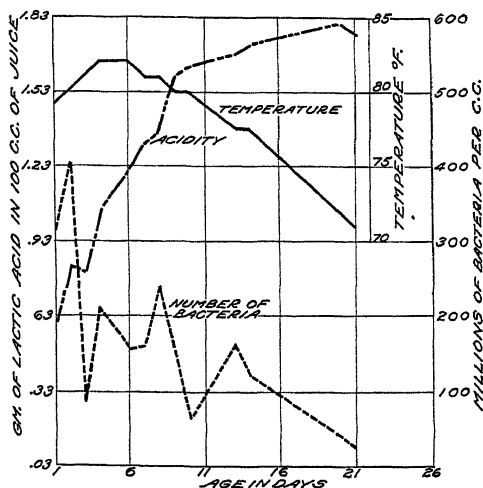


Fig. 10.—Temperature, acidity, and number of bacteria at the center of vat 4 at various times during fermentation.

TABLE 5.—*Changes in number of bacteria in sauerkraut during fermentation*

[C=center sample; B=bottom sample]

[illegible]

## NUMBERS AND KINDS OF BACTERIA

Table 5 and Figures 2 and 4 to 10, inclusive, record the changes in the number of bacteria in each vat during the fermentation. Table 5 includes both bottom and center samples, while the curves give only the changes occurring at the center of each vat. In addition, the curves show the relation between acidity and numbers of bacteria in each vat. From the latter it appears that changes in acidity and numbers of bacteria occur more rapidly in those vats which were filled at the higher temperatures. With the exception of the 40° F. vat there are at least two high points in the bacterial curves. From this fact and also from microscopical evidence it is apparent that there is a sequence of microorganisms taking part in the fermentations. Figure 11, which represents the fermentation in a 10-gallon jar of cabbage kept at 70°, shows this sequence even

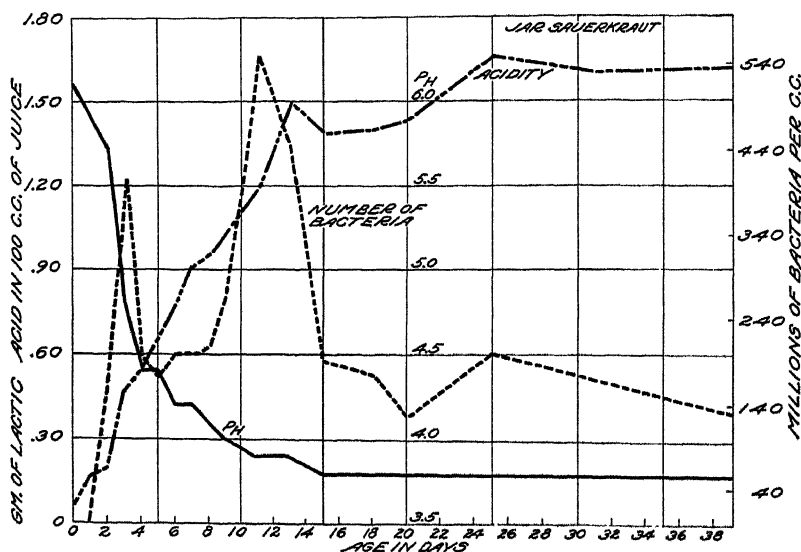


FIG. 11.—Acidity, hydrogen-ion concentration, and number of bacteria in a 10-gallon jar of fermenting cabbage

more clearly. At the first point showing a high number of bacteria, the flora consists of short stubby rods, while at the second point the flora is made up largely of long rods. There are undoubtedly additional types of organisms taking part in the fermentation which are fewer in number and less conspicuous. For example, there are the streptococci occurring during the first few days of fermentation, and the yeast, the importance of which is more or less uncertain.

It is clear that there are at least two types of bacteria taking part in the fermentation. The first is unable to withstand as high concentrations of acid as the second, and is therefore replaced by the latter.

It is worthy of note that although the number of bacteria may rise and fall throughout the fermentation, the increase in acidity is quite uniform. From this it appears that although the organisms die

their enzymes are still active, or else that the new flora which develops upon the death of the old maintains the uniform rate of acid production.

TABLE 6.—Changes in time of reduction of methylene blue by sauerkraut during fermentation

[C=center sample; B=bottom sample; a=not reduced in two hours]

Age of sauerkraut (days)	Time of reduction in minutes in—															
	Vat 2 (initial temperature 44° F.)		Vat 9 (initial temperature 50° F.)		Vat 7 (initial temperature 52° F.)		Vat 5 (initial temperature 53° F.)		Vat 3 (initial temperature 64° F.)		Vat 1 (initial temperature 65° F.)		Vat 6 (initial temperature 74° F.)		Vat 4 (initial temperature 76° F.)	
	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B
1	a	a	a	a	a	a	a	15	80	80	8	9	75	75	8	8
3	a	a	40	40	20	20	40	40	5	5	25	40	5	5	40	40
4	a	a	13	13	12	12	13	95	8	8	34	120	10	10	120	75
5	40	a			40	45			25	28			35	35		
6	11	11	10	10	10	9	20	a	10	10	10	a	8	8	a	20
7			12	12			27	27			27	45			45	45
8	40	40	10	10			55	a	40	40	45	a			a	a
9	22	120	15	90	120	120	a	a	120	75	50	a	120	120	a	a
10			23	65			a	a			50	a			a	a
11	20	a			a	30			10	10			a	a		
12	20	45			45	20			50	27				45		
13	7	a	25	25	a	7	105	a	a	28	a	a			a	a
14	15	a	40	a	a	50	a	45	a	a	a	a				
15	15	a			a	23			a	65						
18	15	a				a			a	45						
19	40	a				8			a	45						
20			a	25			a	40			a	a			a	a
21																
24			45	30			a	45			75	75				
25	40	a			a	40			a	25						
26																
27			105	105			a	135			a	135				
29	75	75			a	120			a	45						
32		165			a	105			a	150						
34																
39																
41	120	120	15	40			120	a								

In Table 6 are recorded the changes in time of reduction of methylene blue of bottom and center samples taken at successive intervals during the fermentations. Figures 12 and 13 show the correlation which exists between the time of reduction of methylene blue and the total number of bacteria in the center samples of two vats. A cold and a warm vat are selected as examples of this relationship, and it is apparent in both cases that when the number of bacteria is high the time of reduction of the dye is short. This inverse relationship seems to indicate that the bacteria shown in microscopic preparations are practically all living organisms.

TABLE 7.—*Changes in acid-forming ability of organisms in sauerkraut during fermentation expressed in percentage of lactic acid*

[C=center sample, B=bottom sample]

Age of sauerkraut (days)	Percentages of lactic acid in—															
	Vat 2 (initial temperature 44° F.)		Vat 9 (initial temperature 50° F.)		Vat 7 (initial temperature 52° F.)		Vat 5 (initial temperature 53° F.)		Vat 3 (initial temperature 64° F.)		Vat 1 (initial temperature 65° F.)		Vat 6 (initial temperature 74° F.)		Vat 4 (initial temperature 76° F.)	
	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B
1	0.33	0.35	0.33	0.49	0.30	0.19	0.32	0.38	0.30	0.32	0.04	0.04	0.30	0.28	0.35	0.10
2	.37	.36	.47	.48	.30	.37	.52	.50	.40	.41	.22	.05	.42	.46	.42	.49
3	.38	.33	.44	.44	.18	.07	.47	.39	.29	.43	.05	.02	.24	.42	.44	.35
4	.18	.13	.05	.07	.17	.07	.06	.06	.21	.28	.03	.16	.24	.19	.13	.28
5	.46	.34			.07	.03			.33	.45			.35	.05		
6	.45	.36	.05	.05	.11	.04			.35	.47			.43	.28	.09	.05
7	.38	.38	.06	.05	.05	.04	.02	.03	.36	.43	.06	.13	.35	.44	.10	.16
8	.01	.13	.19	.00	.23	.14	.06	.08	.12	.17	.02	.02	.14	.15	.12	.00
9	.05	.00	.23	.30	.17	.09	.00	.00	.30	.05	.00	.00	.27	.14	.24	.00
10			.34	.36			.29	.23			.09	.05			.36	.33
11	.05				.14	.11			.02	.03	.11	.22	.13	.38		
12	.16	.23			.02	.04			.23	.24						
13	.34	.39			.33	.23	.29	.29	.20	.33						
14					.42	.35			.37	.36						

## ACID PRODUCTION

In Table 7 are recorded the data for all of the experimental vats, from which samples were taken from day to day and added to fresh cabbage juice. The acid formed in this juice was titrated at the end of a week. When typical examples of these data are plotted, as shown in Figures 14 and 15, it is apparent that there are two points at which the ability of the samples to produce acid is at its highest. These points correspond closely with the high points in the curves showing the number of bacteria.

TABLE 8.—*Composition and grading of sauerkraut from experimental vats (wet basis)*

Age, composition, and quality of sauerkraut	Vat 2 (initial temperature 44° F.)	Vat 9 (initial temperature 50° F.)	Vat 7 (initial temperature 52° F.)	Vat 5 (initial temperature 53° F.)	Vat 3 (initial temperature 64° F.)	Vat 1 (initial temperature 65° F.)	Vat 6 (initial temperature 74° F.)	Vat 4 (initial temperature 76° F.)
Age in days	47	42	39	41	32	28	12	22
Moisture (per cent)	90.7	90.9	90.9	91	90.7	90.5	90.5	91.4
Acetic acid (per cent)	.337	.367	.378	.355	.346	.322	.266	.295
Lactic acid (per cent)	1.242	1.553	1.335	1.367	1.218	1.311	1.062	1.506
Titrateable acid (per cent)	1.377	1.580	1.494	1.600	1.575	1.421	1.410	1.784
Ethyl alcohol (per cent)	.511	.528	.742	.487	.411	.320	.208	.598
Reducing sugar (per cent)	.68	.56	.20	.53	.44	1.05	.76	.35
Quality	Very good.	Excel- lent.	Very good.	Excel- lent.	Poor.	Poor.	Fair.	Fair.

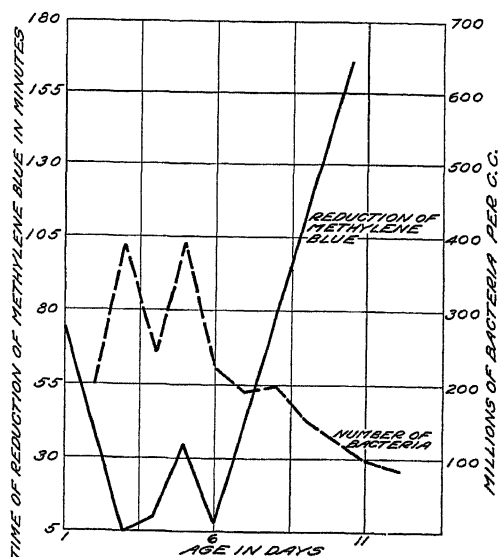


FIG. 12.—Time of reduction of methylene blue and number of bacteria at the center of vat 6 at various times during fermentation

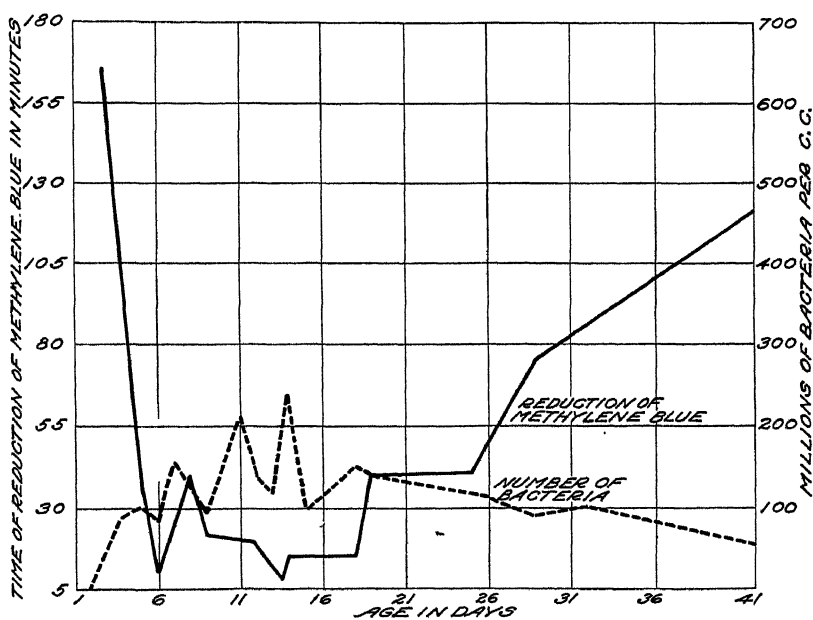


FIG. 13.—Time of reduction of methylene blue and number of bacteria at the center of vat 2 at various times during fermentation

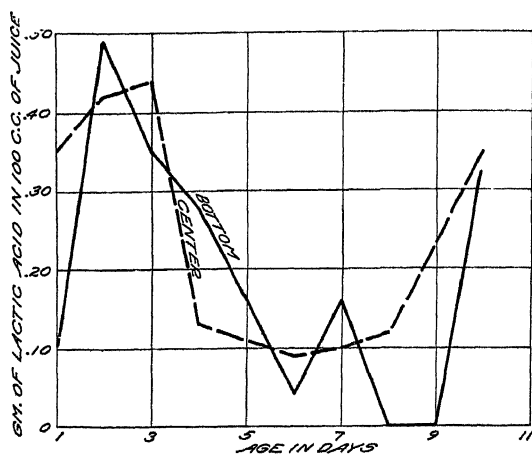


FIG. 14.—Acid-producing ability of samples obtained from the center and bottom of vat 4 at various times during fermentation

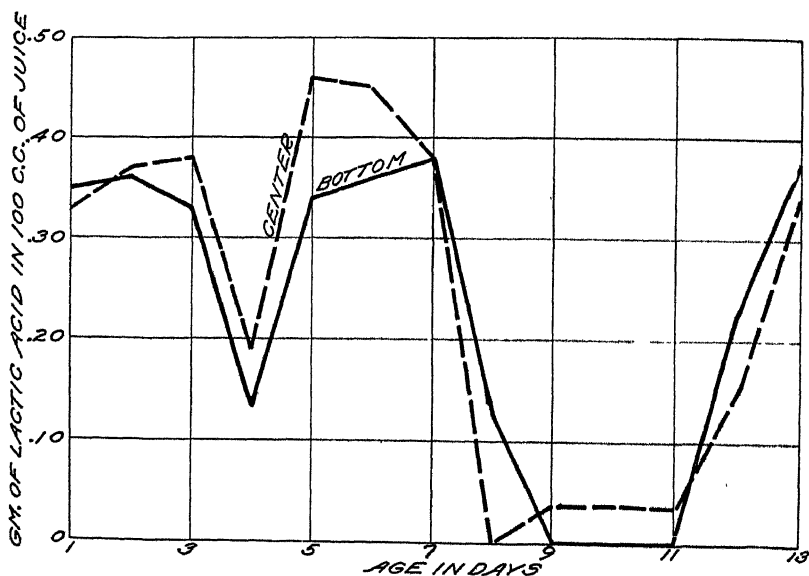


FIG. 15.—Acid-producing ability of samples obtained from the center and bottom of vat 2 at various times during fermentation

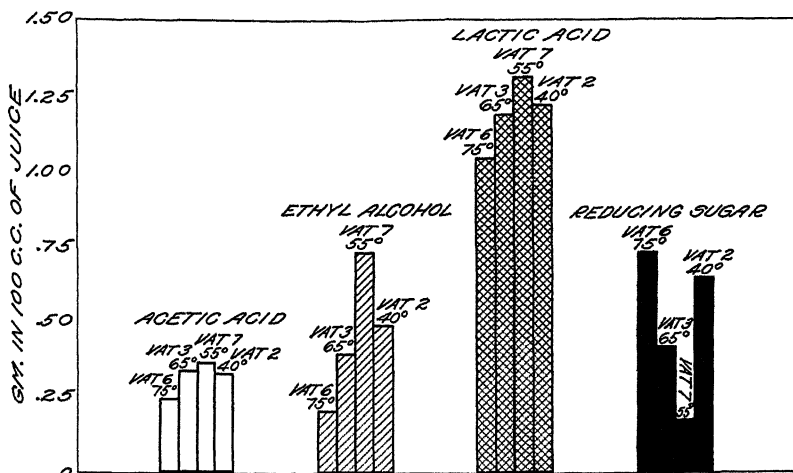


FIG. 16.—Principal fermentation products in sauerkraut obtained from vats 6, 3, 7, and 2

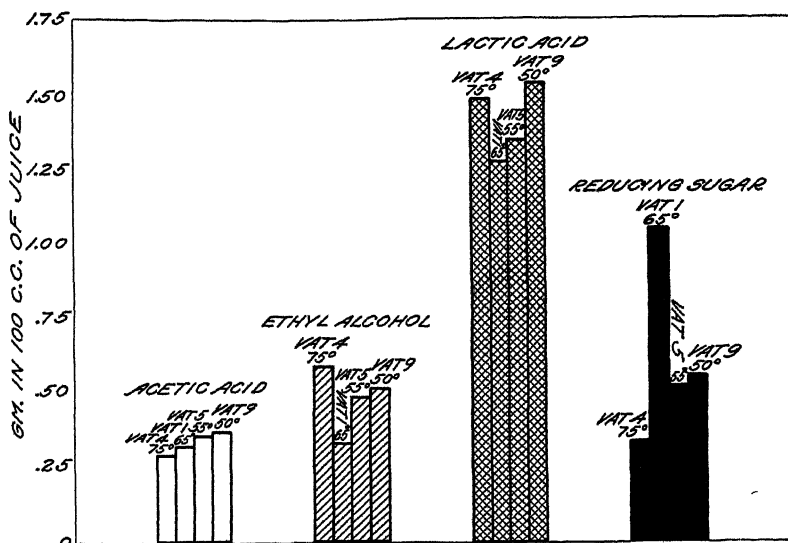


FIG. 17.—Principal fermentation products in sauerkraut obtained from vats 4, 1, 5, and 9

## COMPOSITION AND QUALITY OF SAUERKRAUT

Figures 16 and 17 and Table 8 show the composition of the experimental sauerkraut in the two series of vats. The best sauerkraut was obtained from the vats at the intermediate temperatures; at higher temperatures the product was more or less pink and of poor quality. Presumably the fermentation at these temperatures went on too rapidly, affording conditions favorable for the growth of pink yeasts. It is difficult to observe any relation between composition and quality of sauerkraut from the data at hand. Undoubtedly the flavor is influenced by minute quantities of unidentified products of fermentation. As is to be expected in almost every case the amount of fermentation products formed is inversely proportional to the residual sugar.

## SUMMARY AND CONCLUSIONS

Evidence is presented which indicates that heat is produced during the fermentation of cabbage.

The rate of acid production in fermenting cabbage is largely dependent upon the temperature of the fermentation. The higher the temperature of fermentation the greater the rate of acid production.

Titrateable acidity is a much better measure of the degree of fermentation than are  $P_H$  determinations.

The fermentation of cabbage is carried on by a sequence of flora, consisting of at least two distinctly different types of lactic acid-producing bacteria.

The time of reduction of methylene blue is of value as an indication of the number of live bacteria in fermenting cabbage.

Sauerkraut of the best quality was produced when the maximum temperature of fermentation was about 65° F.

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# STRAIN VARIATIONS AND HOST SPECIFICITY OF THE ROOT-NODULE BACTERIA OF THE PEA GROUP<sup>1</sup>

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## INTRODUCTION

Since early in the study of the root-nodule bacteria, observations have been made indicating that variations may exist between strains of bacteria within a cross-inoculation group, and that such differences may be closely related to the nitrogen economy of the plant. The generally accepted opinion, however, has been that the strains of bacteria of a cross-inoculation group are practically identical as to their ability to aid the plant in the symbiotic fixation of nitrogen. Cultures distributed by public agencies and commercial concerns have been selected on the basis of the cross-inoculation group. Probably some strains are equally good on more than one species and possibly on all the members of their group, but there seem to be other strains of the nodule organism which show a specificity for a single host species, and which are of great benefit to one member of the host group, but of relatively little benefit to another member.

Even though certain strains show a preference for a single host species, it is scarcely probable that all of the strains isolated from that species would exhibit the same peculiarity. When an organism is isolated from a host plant it is usually impossible to determine its previous history, and it is possible that a strain isolated from a pea nodule may have been associated with vetch or some other host of the cross-inoculation group within a few previous generations.

## REVIEW OF LITERATURE

As early as 1879, Frank (5)<sup>2</sup> recognized two types of inoculation on peas, one type forming nodules with albuminous contents, the other forming nodules containing amyloextrin. The albuminous ones contained 6.93 per cent of nitrogen in the dry matter as compared to 4.82 per cent of nitrogen in the dry matter of those containing amyloextrin.

Beyerinck, 1888 (1), described two types of nodules: One in which the bacteria gained the ascendancy and destroyed the interior, they themselves remaining alive; the other, in which the nodule i. e., the host plant, received the advantage, the bacteria being converted mostly into bacteroids, furnishing food for the plant but incapable of growth.

Nobbe and Hiltner, 1893 (12), inoculated peas with several pure cultures one of which gave particularly poor results, the plants being inferior in size and color to the others. The nodules produced by this culture were very numerous and large, but contained few bacteroids. This apparently was the condition which Hiltner in a later paper, 1904 (8), described as the fifth type of immunity, i. e., the organisms are so efficient in comparison with the plant that the latter is injured.

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<sup>2</sup> Reference is made by number (*italic*) to "Literature cited," p. 1053.

In another paper Nobbe, Hiltner, and Schmid, 1895 (13), reported some work on the efficiency of cultures in cross inoculations. Organisms from *Robinia pseudacacia*, *Acacia lophanta*, *Vicia sepium*, and *Pisum* were used to inoculate *Robinia*, *Acacia*, and *Vicia villosa*. All four cultures produced nodules on both the *Robinia* and *Acacia* plants. The *Robinia* culture on *Robinia* and the *Acacia* culture on *Acacia* produced very large nodules; the other inoculations produced very small ones. The vetch did not form nodules when inoculated with either *Robinia* or *Acacia* cultures. Both the vetch and pea cultures formed nodules on vetch, the vetch inoculation producing large nodules on the secondary and tertiary roots, and the pea inoculation very much smaller nodules on the tertiary and quaternary roots. A summary of the dry weights and nitrogen percentages of this experiment is given in Table 1. From three such experiments in nitrogen-free sand, Nobbe, Hiltner, and Schmid (13) concluded that the various leguminous plants were benefited most readily if inoculated with bacteria from nodules of their own species.

TABLE 1.—*Plant weights and nitrogen percentages obtained by inoculating Robinia, Acacia, and Vicia Villosa with various cultures* \*

Source of bacteria	Robinia		Acacia		Vicia villosa	
	Dry weight	Nitrogen in tops	Dry weight	Nitrogen in tops	Dry weight	Nitrogen in tops
	Gm.	Per cent	Gm.	Per cent	Gm.	Per cent
Robinia.....	7.4020	3.23	1.953	0.85	0.7829	1.40
Acacia lophanta.....	1.1582	1.77	6.943	1.44	.8658	1.59
Vicia sepium.....	.8584	1.45	1.248	1.05	9.1331	2.87
Pisum.....	1.4795	1.40	1.817	.92	1.0334	2.22

\* From Nobbe, Hiltner, and Schmid

In analyzing the above data it must be remembered that this work was carried out before many of the later methods for the determination of pure cultures were devised or before cross-inoculation limitations were fully recognized. The differences obtained by the inoculation of vetch with the vetch and pea organisms, however, probably demonstrate a strain variation.

In 1900, Hiltner (7) reported some work indicating strain variations. Filtered *Pisum* nodule extract was applied to both *Pisum* and *Lathyrus* roots. The extract apparently contained some substance which caused a curling of the root hairs similar to that occurring with an invasion of the nodule organism. The curling of the *Lathyrus* however, was much slower, indicating that the organism in the pea nodule was more specific for peas (*Pisum*) than for sweet peas (*Lathyrus*).

Dehérain and Demoussy, 1900 (4), working with the white lupin concluded that the amount of nitrogen fixed by it depended on the types of nodules produced. They distinguished four types of nodules:

1. Small nodules placed on the main roots resembling a string of beads. The nitrogen-fixing ability of this form was high, the plants containing about 3 per cent nitrogen in the dry matter.

2. Medium-size smooth nodules, sometimes forming a collar about the top of the root. Plants with this type of nodule contained about 2 per cent nitrogen in the dry matter.

3. Half spherical nodules sometimes encasing the roots (vetch) or sometimes projecting from the roots (alfalfa). The plants having this form contained about 1 per cent nitrogen in the dry matter.

4. Enormously large nodules in the form of a raspberry. Plants of this type contained only 0.6–0.8 per cent nitrogen in the dry matter.

Moore, 1905 (11), made the following statement regarding the ability of the plant to receive help from certain types of nodules:

Thus, if nodule-forming organisms be grown upon artificial media for a long time, where they are almost invariably in the rod condition, this form becomes so firmly established that plants inoculated with such cultures, although forming nodules, receive practically no benefit, the nodules remaining firm and hard and furnishing no nitrogen to the roots.

Harrison and Barlow, 1907 (6), discussing cross inoculation between pea and vetch, state:

Cross inoculations have not been observed. Pea and vetch growing in the same flask, which was inoculated with vetch culture, formed abundant nodules and root infection occurred in the vetch, but the pea showed no infection whatever.

This is difficult to explain in the light of present knowledge, as pea and vetch are known to cross inoculate. There is a possibility, however, that Harrison and Barlow had a strain which inoculated the vetch only.

New York (Cornell) Experiment Station, 1918 (10), made a preliminary report on the efficiency of different strains of *Bacillus radicicola* in the production of nodules and the fixation of nitrogen when transferred from the true hosts to secondary ones. They stated that the organisms of peas transferred to vetch produced nodules, but without nitrogen fixation. No further report of this work has been found.

Bialosuknia, 1923 (2), from the results of his investigations drew the following conclusions in regard to host-plant specificity:

1. Bacteria of the pea in passing to broad bean form nodules but give a minimum effect.

2. This relationship is not changed by two plant passages.

Stevens, 1925 (14), working with various strains of the alfalfa root-nodule organism concluded:

Strains of the alfalfa, sweet clover group of nodule bacteria differ under similar conditions in their ability to fix free nitrogen and to benefit the host plant.

He was able to show differences in the cultural, physiological, and serological characteristics of the various strains.

Wright, 1925 (15, 16), demonstrated two types of soy-bean nodule organisms which varied greatly in their cultural, physiological, and serological properties. These types also differed widely in their ability to aid the plant in the fixation of nitrogen.

In addition to the observations reported in the literature differences were noticed in type of nodule development and plant growth in both greenhouse and field, due to the use of different strains of bacteria on peas. As a result of the above observations, experiments were carried out to study further the questions of strain variations and host-plant specificity.

## MATERIAL AND METHODS

Seventeen strains of nodule bacteria were used; however, a major portion of the work was done with only a few of the strains which showed significant differences. These strains were all Wisconsin stock cultures of various ages, none of which had been through a plant passage since their addition to the Wisconsin culture collection. A summary of the histories of these strains is given in Table 2. It should be noted that strain 15 was secured by picking an isolated colony from a plate made of strain 8. Immediately before the experiments were begun, the cultures were tested twice for purity on milk and potato media, according to the method of Löhnis and Hansen 1921 (9). They were also tested three times for nodule formation and were all found to be efficient. During these preliminary tests differences in the type of nodule production were noticed.

TABLE 2.—History of cultures used in experiments 1 to 5

Wisconsin strain No.	Year secured	Source
2	1922	Isolated from a commercial pea culture.
3	1921	Isolated from pea nodule at Madison, Wis.
4	1921	Secured from University of Illinois, isolated from pea nodule.
5	1924	Isolated from pea nodule (Late Sweets), Urbana, Ill.
6	1918	Secured from U. S. Department of Agriculture, pea culture.
7	1918	Isolated from a commercial pea culture.
8	1916	Secured from U. S. Department of Agriculture, vetch culture.
9	1922	Isolated from commercial vetch culture.
10	1924	Isolated from pea nodule (Late Sweets), Urbana, Ill.
11	1924	Do.
13	1926	Secured from U. S. Department of Agriculture, Vicia faba culture.
14	1926	Secured from Kluyver (Holland) pea culture.
15	1924	Secured from University of Illinois; original source U. S. Department of Agriculture, vetch culture.*
20	1924	Isolated from pea nodule (Alaska pea), Urbana, Ill.
21	1924	Do.
22	1924	Do.
23	1924	Do.

\* This culture was secured at the University of Illinois by replating the U. S. Department of Agriculture vetch culture numbered Wisconsin 8, above.

With the exception of the first experiment, the plants were grown on glacial sand. This sand was found to be very low in nitrogen, and as a result differences in nitrogen fixation were plainly evidenced in plant growth. The pots were covered with paper after being filled with sand and autoclaved at 15 pounds for 16 to 20 hours. The seed used in these experiments was freed of the nodule organism by immersion in hot water at 65° C. for four minutes. The plants were watered whenever necessary with sterile distilled water. An application of liquid culture solution was made every two weeks. The solution used was a modification of Bryan's, 1922 (3). The formula follows:

KCl.....	10. 0 gm.
CaSO <sub>4</sub> .....	2. 5 gm.
MgSO <sub>4</sub> .....	2. 5 gm.
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> .....	2. 5 gm.
FePO <sub>4</sub> .....	2. 5 gm.
K <sub>2</sub> PO <sub>4</sub> .....	2. 5 gm.

The salts were ground together in a mortar and used at the rate of  $1\frac{1}{2}$  gm. per liter of distilled water. After shaking, the solution was allowed to stand 24 hours. The clear solution was then decanted and sterilized.

## EXPERIMENTAL DATA

### EXPERIMENT 1

#### GROWTH OF GREEN ADMIRAL PEAS IN NITROGEN-FREE SAND, INOCULATED WITH VARIOUS CULTURES

The white quartz sand used in this experiment was treated with hydrochloric acid until all nitrates had been leached out. It was then washed thoroughly with distilled water and made up for each pot as follows:

Nitrate-free sand.....	2, 500 gm.
Nitrate-free kaolin.....	10 gm.
Nitrate-free $\text{CaCO}_3$ .....	1 gm.
Distilled water.....	275 c.c.

After sterilization, nine pots were planted with seeds inoculated with each strain. Six strains were used in this experiment. After two months' growth the plants were washed out of the pots for observation and analysis. The results are given in Table 3. The growing conditions during this experiment were very poor, the weather being too hot for the growth of peas. The results, however, are interesting since they show a wide variation in the nitrogen content of the plants inoculated with different cultures. Plants inoculated with strain 15 were especially low in nitrogen, suggesting that this strain fixes but little nitrogen.

TABLE 3.—*Dry weight and nitrogen content of Green Admiral peas inoculated with various cultures, and grown in the greenhouse in nitrogen-free sand; April, 1926*

Inoculated with strain No.—	Average weight per plant air dry	Nitrogen in water-free material	Total nitrogen per plant	Inoculated with strain No.—	Average weight per plant air dry	Nitrogen in water-free material	Total nitrogen per plant
	Gm.	Per cent	Mgm.		Gm.	Per cent	Mgm.
2.....	0.308	2.10	0.6468	15.....	0.333	1.88	0.6260
4.....	.330	2.36	.7788	20.....	.328	2.31	.7577
6.....	.376	2.01	.7558	Not inoculated.....	.336	1.80	.6048
10.....	.312	2.05	.6396				

### EXPERIMENT 2

#### GROWTH OF GREEN ADMIRAL PEAS IN SAND, INOCULATED WITH VARIOUS CULTURES

The peas grown in glacial sand showed significant differences. (Table 4.) The uninoculated plants and those inoculated with strain 15 were of a yellowish-green color and very much shorter than the other plants. The plants inoculated with strain 13 also did not have a normal dark-green color, but were slightly tinted with yellow.

TABLE 4.—Color, dry weight, and nodule formation of Green Admiral peas inoculated with various cultures and grown in the greenhouse in glacial sand; April, 1927

Inoculated with strain No.—	Color of vines	Average weight per plant air dry	Type of nodule formation
		<i>Gm.</i>	
2.....	Normal green.....	0.900	Large nodules on taproot near surface of soil.
3.....	do.....	.815	Similar to No. 2.
4.....	do.....	.913	Large nodules on taproot near surface, also some scattered on lower roots.
5.....	do.....	.900	Similar to No. 4.
6.....	do.....	.993	Do.
7.....	do.....	1.044	Do.
8.....	do.....	.971	Similar to No. 2.
9.....	do.....	1.011	Do.
10.....	Pale green.....	.822	Similar to No. 4, but nodules somewhat smaller.
11.....	Normal green.....	.880	Similar to No. 4.
13.....	Pale green.....	.777	Nodules fairly well scattered over roots; many small ones.
14.....	Normal green.....	.911	Similar to No. 2.
15.....	Yellowish green.....	.400	Very small nodules scattered throughout roots.
20.....	Normal green.....	.955	Similar to No. 4.
21.....	do.....	.811	Do.
22.....	do.....	.840	Do.
23.....	do.....	.705	Do.
Not inoculated.....	Yellowish green.....	.314	An occasional nodule; located anywhere on roots.

It is interesting to note that a relationship seems to exist between the position of the nodules on the roots and the benefit derived by the plant. The cultures which were of greatest benefit to the plant produced large nodules, practically all of which were located on or near the primary root and near the surface of the soil. Strain 15 produced a large number of nodules, all of which were exceedingly small. These were scattered over the entire root system, as many appearing on the lower as on the upper roots. Strain 13, and to a lesser extent strain 10, produced medium-size nodules which were scattered over the roots.

The uninoculated plants and the strain 15 inoculated plants produced only a few pods which contained 2 or 3 peas per pod. All other plants produced several large pods containing 5 and 6 peas per pod.

#### EXPERIMENTS 3 AND 4

##### GROWTH OF ALASKA PEAS IN SAND, INOCULATED WITH VARIOUS CULTURES

The plants in experiment 3 were harvested and the observations recorded (Table 5) after six weeks' growth. The differences in growth are shown in Figure 1. The pots are numbered with the number of the strain used in inoculating them. The plants which showed a pronounced yellowish color are the uninoculated, No. 12 and No. 15. Plants inoculated with strain 13 did not grow as well as the others, but had a healthy green color. Pot 12 was inoculated with a *Bacillus radiobacter* culture and the plants produced no nodules. The difference in color of plants inoculated with strain 15 and those inoculated with the other strains is well defined. Although numerous nodules were produced on the roots of these plants, there was apparently no benefit received as the plants did not develop any further than the controls.

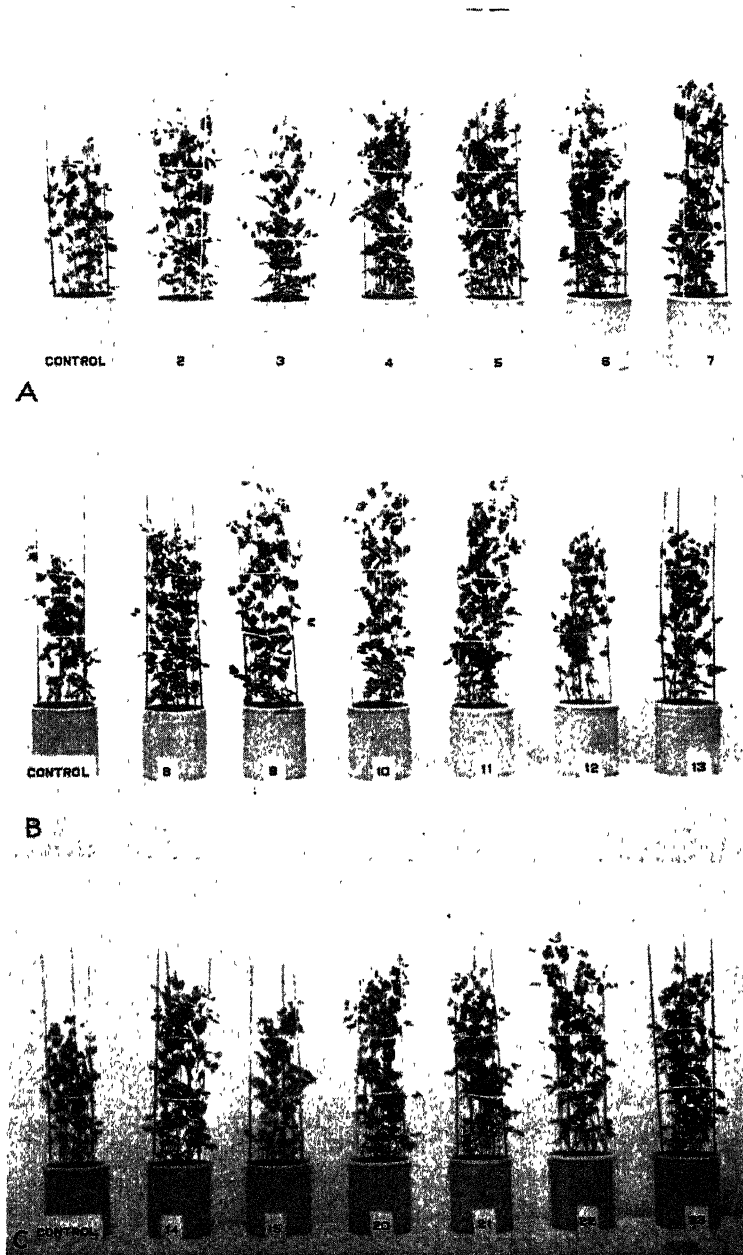


FIG. 1.—Variations in the growth of Alaska peas (*Pisum sativum*) induced by inoculation with various strains of bacteria: A, Pots containing plants uninoculated and inoculated with strains 2, 3, 4, 5, 6, and 7. B, Pots containing plants uninoculated and inoculated with strains 8, 9, 10, 11, 12, and 13. C, Pots containing plants uninoculated and inoculated with strains 14, 15, 20, 21, 22, and 23

TABLE 5.—*Color, height, dry weight, and nodule formation of Alaska peas inoculated with various cultures and grown in the greenhouse in glacial sand; February, 1927*

Inoculated with strain No.—	Color of vines	Height of plant	Average weight per plant air dry	Type of nodule formation
		<i>Inches</i>	<i>Gm.</i>	
2.....	Normal green.....	18	0.754	Large nodules on taproot near surface of soil.
3.....	do.....	18	.588	Similar to No. 2.
4.....	do.....	19	.673	Many nodules on taproot and scattered on upper roots.
5.....	do.....	20	.683	Similar to No. 4.
6.....	do.....	21	.664	Do.
7.....	do.....	20	.759	Do.
8.....	do.....	19	.636	Similar to No. 2.
9.....	do.....	20	.690	Do.
10.....	do.....	20	.646	Nodules somewhat smaller and more scattered than No. 4.
11.....	do.....	19	.575	Similar to No. 4.
13.....	do.....	17	.606	Nodules somewhat smaller and more scattered than No. 4.
14.....	do.....	21	.572	Similar to No. 4.
15.....	Yellowish green.....	14	.535	Many very small nodules scattered all over roots.
20.....	Normal green.....	21	.553	Similar to No. 4.
21.....	do.....	20	.541	Do.
22.....	do.....	20	.570	Do.
23.....	do.....	22	.562	Do.
Not inoculated.....	Yellowish green.....	13	.539	One pot of six contained a few nodules.

The nodules on the plants inoculated with strain 15 differed greatly from those on the other plants. The nodules were very small and scattered very low on the roots. (Fig. 2, A.) Practically no nodules formed on the taproot. Most of the nodules appeared undeveloped. The roots inoculated with strain 3 had large nodules on the taproot and upper side roots. (Fig. 2, B.) Practically no nodules appeared low in the roots. The nodules produced by all strains except 10, 13, and 15 were very similar to this type. Strains 10 and 13 gave nodules which seemed to be intermediate in type between Nos. 3 and 15.

Experiment 4 was a repetition of Experiment 3, and the results were very similar. (Table 6.) All of the cultures except strain 15 showed increases in growth over the controls. The plants inoculated with strain 15 produced even less dry weight than the controls. This, however, may have been due to the fact that the controls formed a few nodules, apparently from air contamination.

TABLE 6.—*Color, dry weight, and nodule formation of Alaska peas inoculated with various cultures and grown in the greenhouse in glacial sand; February, 1927. Duplicate of experiment shown in Table 5*

Inoculated with strain No.—	Color of vines	Average weight per plant air dry	Type of nodule formation
		<i>Gm.</i>	
2.....	Normal green.....	1.073	Large nodules practically all on taproot.
3.....	do.....	1.110	Similar to No. 2.
4.....	do.....	1.128	Nodules on taproot and scattered in upper roots.
5.....	do.....	1.053	Similar to No. 4.
6.....	do.....	1.063	Do.
7.....	do.....	1.119	Do.
8.....	do.....	1.146	Do.
9.....	do.....	1.052	Similar to No. 2.
10.....	do.....	1.044	Nodules somewhat smaller and more scattered than No. 4.
11.....	do.....	1.054	Similar to No. 2.
13.....	Pale green.....	.922	Similar to No. 10.
14.....	Normal green.....	.932	Similar to No. 2.
15.....	Yellowish green.....	.647	Very small nodules scattered throughout root system.
20.....	Normal green.....	.920	Similar to No. 4.
21.....	do.....	.927	Do.
22.....	do.....	.950	Do.
23.....	do.....	.929	Do.
Not inoculated.....	Yellowish green.....	.729	An occasional nodule.

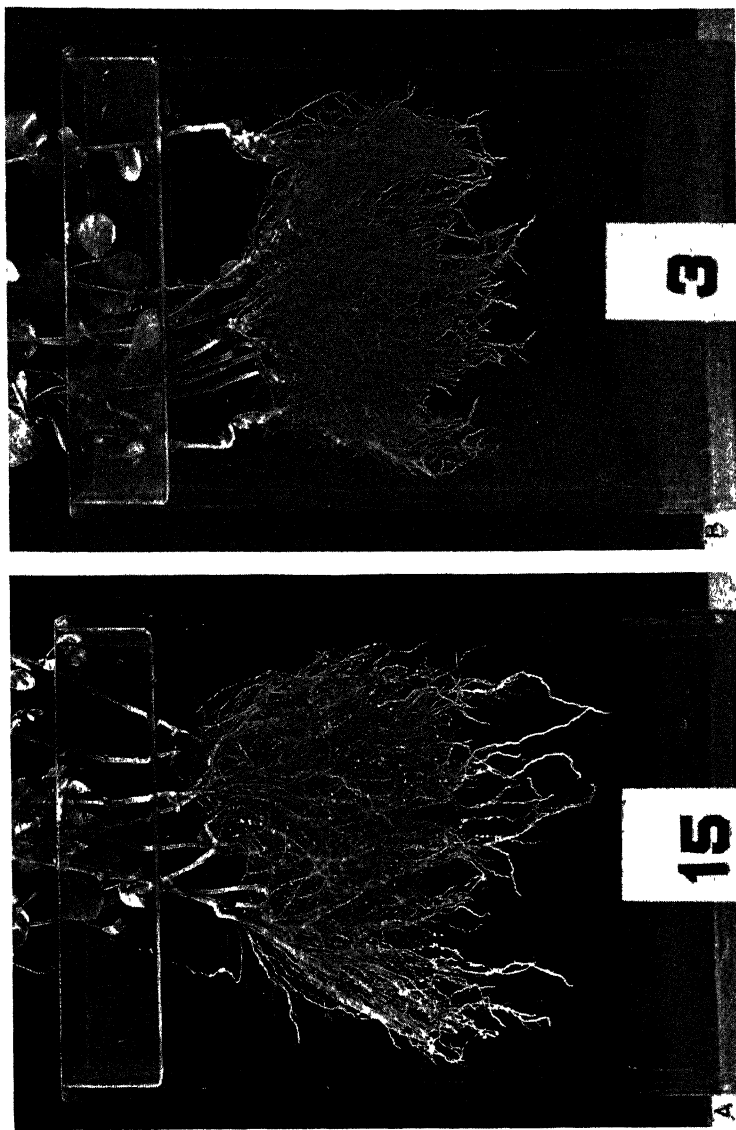


FIG. 2.—Roots of Alaska peas (*Pisum sativum*) showing type of nodule formation: A, Inoculated with strain 15. Numerous small nodules, scattered all over the root system. B, Inoculated with strain 3. Relatively few large nodules, placed almost exclusively on the upper parts of the root system.

## EXPERIMENT 5

GROWTH OF *VICIA FABA*, *LATHYRUS ODORATUS*, *VICIA VILLOSA* AND *LENS ESCULENTA* IN SAND, INOCULATED WITH VARIOUS CULTURES

The fact that strain 13, which was known to have been isolated from *Vicia faba*, did not give as good results on peas as the pea cultures brought up the question of whether it would benefit its own original host plant more than would a pea culture.

*VICIA FABA*

*Vicia faba* was, therefore, inoculated with strains 13 and 20. The plants inoculated with strain 20 (Table 7) were somewhat better than the controls, but similar to them, both having wilted and dried lower leaves. (Fig. 3, A.) The *Vicia faba* culture, strain 13, produced a very good growth of plants, the dry matter produced being greater than that of the plants inoculated with strain 20.

The nodules produced by strain 20 (fig. 4, A) were very small, and similar to those of the lupin type. They were fairly well scattered over the root system. The nodules produced by strain 13 (fig. 4, B) were very large and well formed, being placed on the upper roots near the surface of the soil. The roots of a plant from an uninoculated pot (fig. 4, C) contained no nodules.

TABLE 7.—Color, dry weight, and nodule formation of broad bean inoculated with strains 13 and 20 and grown in the greenhouse in glacial sand; February, 1927

Inoculated with strain No. —	Color of vines	Average weight per plant air dry	Type of nodule formation
13.....	Normal green; all leaves healthy..	Gm. 0.077	Many large, well-formed nodules.
20.....	Pale green; lower leaves dried.....	.066	Many small, scattered, underdeveloped nodules.
Not inoculated...	Yellowish green; lower leaves dried.	.049	No nodules.

*LATHYRUS ODORATUS*

On sweet peas as on garden peas strain 15 gave very inferior results. (Table 8.) It appeared that the plants were not only suffering from a nitrogen deficiency, but that the organisms on the roots were actually parasitic. The dry weight of the plant inoculated with strain 15 was even less than the weight of the uninoculated plants.

TABLE 8.—Color, height, dry weight, and nodule formation of sweet peas inoculated with various cultures and grown in the greenhouse in glacial sand; April, 1927

Inoculated with strain No.—	Color of vines	Height of plants	Average weight per plant air dry	Type of nodule formation
3.....	Normal green.....	Inches 20-26	Gm. 1.363	Large well-formed nodules on taproot near surface of soil.
13.....	Pale green.....	16-18	.488	Fair-sized nodules on upper roots; some scattered low in roots.
15.....	Yellowish green...	4-8	.310	Very small nodules scattered throughout root system.
20.....	Normal green.....	20-30	1.268	Large well-formed nodules on taproot near surface of soil.
Not inoculated...	Yellowish green...	6	.429	No nodules.

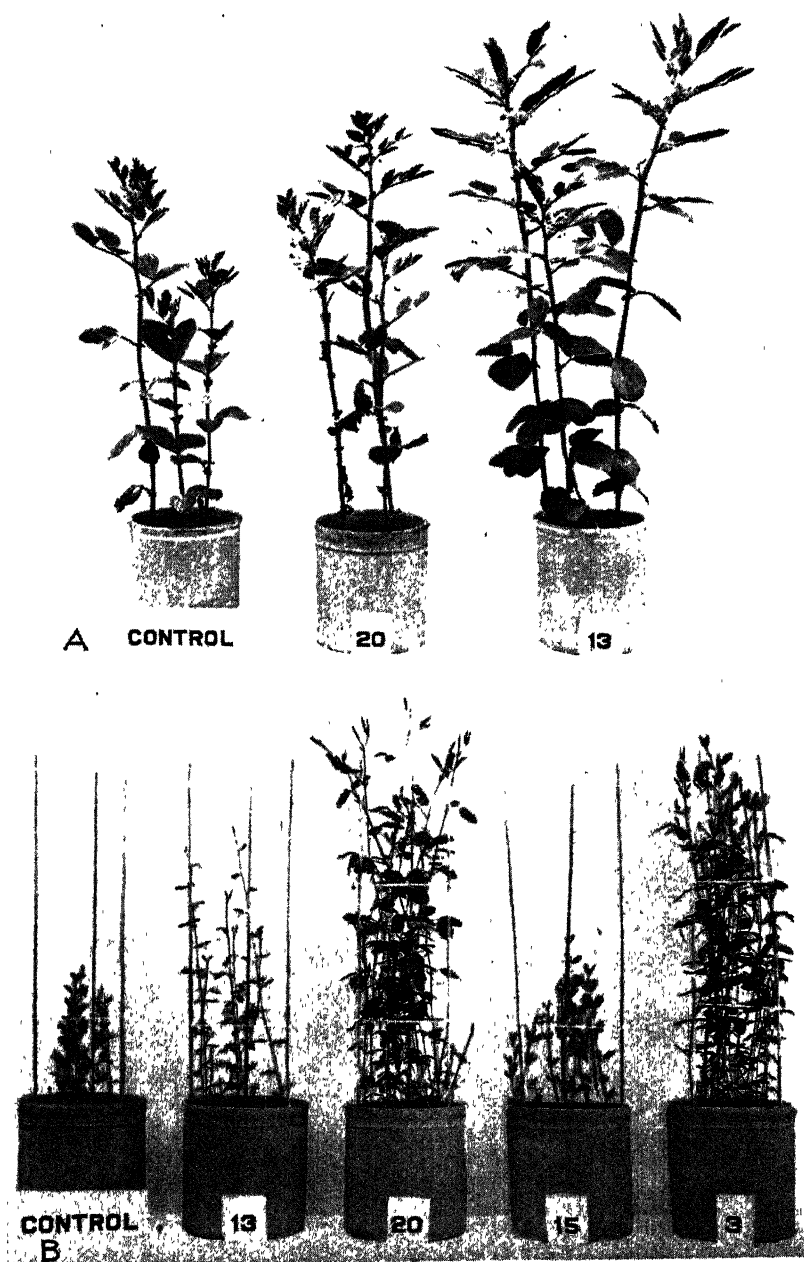


FIG. 3.—Variation in plant growth induced by inoculation with various strains of bacteria: A, Broad bean (*Vicia faba*). Plants inoculated with strain 13 have made better growth than those inoculated with strain 20. B, Sweet pea (*Lathyrus odoratus*). Plants inoculated with strain 20 have made much better growth than those inoculated with strain 13.

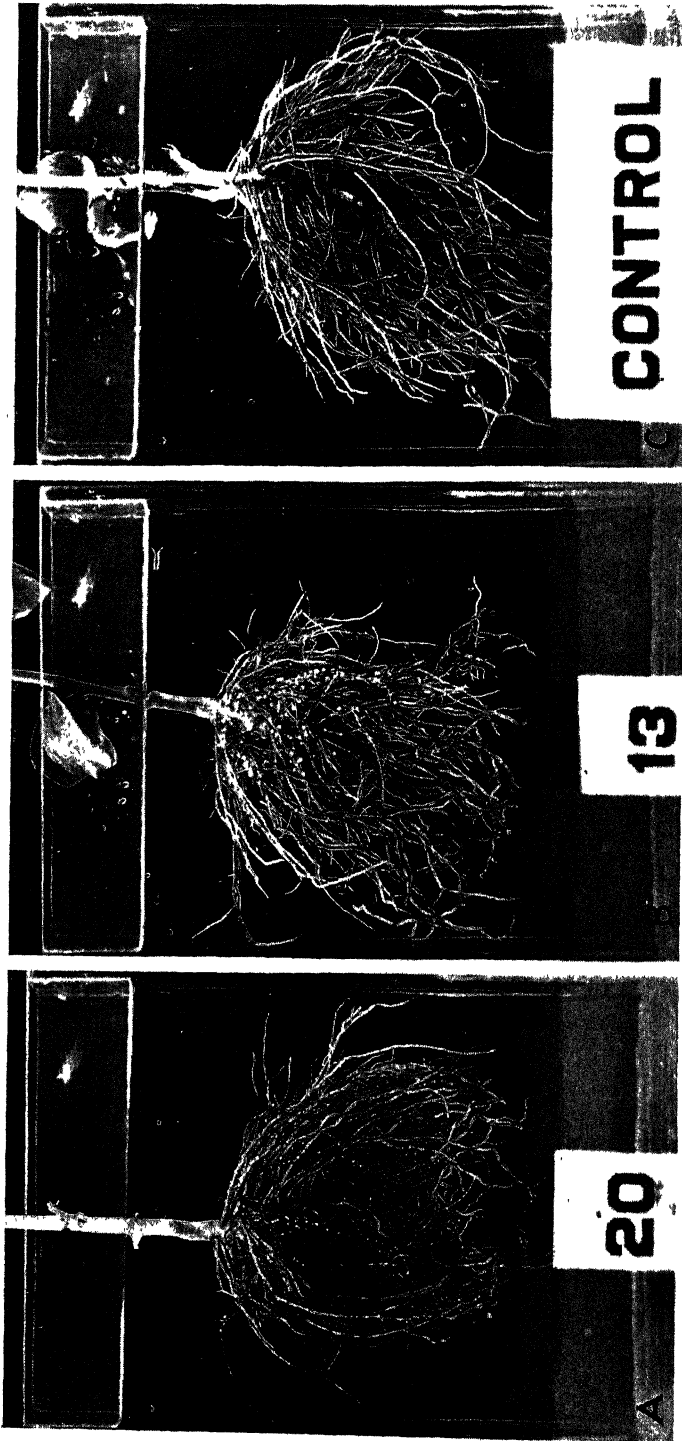


FIG. 4.—Roots of broad beans (*Vicia faba*) showing type of nodule formation: A, Inoculated with strain 20. Numerous small nodules, scattered over the entire root system. B, Inoculated with strain 13. Relatively few large nodules, placed on the upper parts of the root system. C, Uninoculated. No nodules.

The plant growth of the sweet peas was much influenced by the use of different cultures. (Fig. 3, B.) The plants inoculated with strain 15 were yellowish light green similar to the controls. Plants inoculated with strain 13, although producing more dry matter, were very thin and weak. Strains 3 and 20 gave excellent dark green plants.

The nodules on the plants inoculated with strain 13 (fig. 6, A) were of medium size and scattered fairly well on the side roots near the top of the plant. The roots inoculated with strain 15 (fig. 6, B) had exceedingly small nodules scattered throughout the root system, many being located low on the roots. The nodules produced by strain 20 (fig. 6, C) were large and located on the taproot and upper side roots near the taproot.

## VICIA VILLOSA

Differences produced in the growth of hairy vetch inoculated with various cultures are given in Table 9. Strain 13 apparently produced just as good a growth of vetch, as did either strains 3 or 20. (Fig. 5, A.) The nodule placings on plants inoculated with strains 3, 13, and 20 were very similar. Strain 15, however, produced much smaller nodules somewhat lower on the roots.

TABLE 9.—*Color, dry weight, and nodule formation of hairy vetch inoculated with various cultures and grown in the greenhouse in glacial sand; April, 1927*

Inoculated with strain No.—	Color of vines	Average weight per plant air dry	Type of nodule formation
3.....	Normal green....	Gm. 0.375	Large well-formed nodules scattered on roots.
13.....	do.....	.395	Do.
15.....	Yellowish green....	.150	Very many small nodules scattered on roots.
20.....	Normal green....	.343	Large well-formed nodules scattered on roots.
Not inoculated....	Yellowish green....	.163	None.

## LENS ESCULENTA

The results obtained in the growth of the lentil are given in Table 10. Figure 5, B, shows the relative differences in growth. The results were very similar to those obtained with the vetch, strain 15 being the only one which did not show a great benefit to the plant.

TABLE 10.—*Color, dry weight, and nodule formation of lentil inoculated with various cultures and grown in the greenhouse in glacial sand; April, 1927*

Inoculated with strain No.—	Color of vines	Average weight per plant air dry	Type of nodule formation
3.....	Normal Green....	Gm. .735	Many large nodules in upper roots.
13.....	do.....	.626	Many fairly large nodules in upper roots.
15.....	Yellowish green....	.450	Many very small nodules scattered throughout roots.
20.....	Normal green....	.663	Many large nodules on tap and upper roots.
Not inoculated....	Yellowish green....	.455	An occasional nodule.

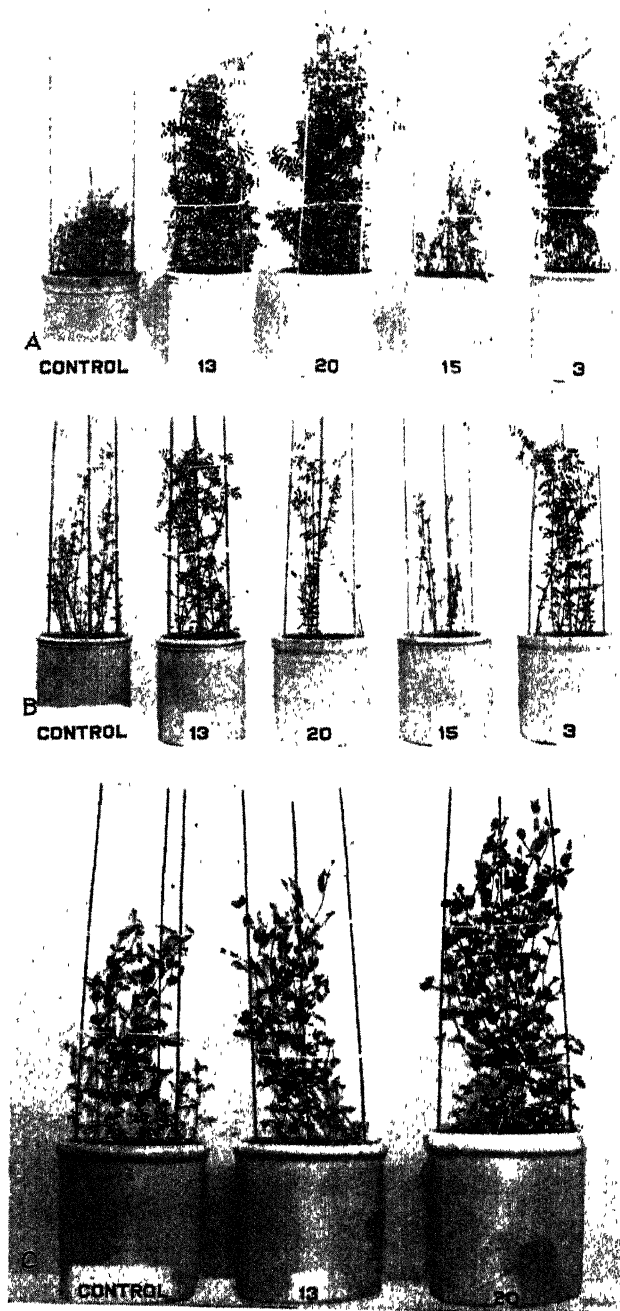


FIG. 5.—Variation in plant growth induced by inoculation with various strains of bacteria: A, Hairy vetch (*Vicia villosa*). Plants inoculated with strains 13 and 20 have made about equal growth. B, Lentil (*Lens esculenta*). Plants inoculated with strains 13 and 20 have made about equal growth. C, Green Admiral Peas (*Pisum sativum*). Plants inoculated with strain 20 have made better growth than those inoculated with strain 13.

## DISCUSSION

The fact that strain variations may exist which affect the ultimate benefit derived by the plant is clearly demonstrated. A nodule organism which originally was isolated from *Vicia faba* (strain 13) gave excellent plant growth when used with *V. faba* in comparison with a culture (strain 20) originally isolated from pea. (Fig. 3, A.) This relationship was exactly reversed when the two cultures were used to inoculate peas, the pea strain (No. 20) giving the better results. (Fig. 5, C.) On vetch and on lentil the *V. faba* strain apparently was as efficient as any of the pea strains. On sweet peas, however, a great difference was again shown, the inoculation with the pea cultures giving very good results in comparison with the *V. faba* culture.

The location of the nodules in a root system and the size of the nodules seem to give an indication of the effectiveness of a culture. Strain 13 on *Vicia faba* (fig. 4, B) produced abundant, large nodules in the upper roots. This culture on sweet peas (fig. 6, A) produced smaller nodules scattered over the root system. With strain 20 the reverse took place. The nodules on the sweet pea were located on the taproot and other upper roots (fig. 6, C), while those on the *V. faba* were scattered throughout the root system (fig. 4, A). In general, throughout this work the cultures which were beneficial to their host plants produced large nodules on the taproot or other upper roots of the plant. Those cultures which were not so beneficial formed small nodules scattered throughout the root system.

In each of the experiments on all of the host species strain 15 was shown to be unable to aid the plant. Although an abundance of nodules was always formed, the plants never grew any better than the uninoculated plants. In fact, the growth of the plants inoculated with strain 15 was often poorer than that of plants having no nodules. The possibility exists that this organism, though able to stimulate the plant in the formation of nodules, is yet unable to fix nitrogen, and that any nitrogen going into the make-up of the organism is derived from the plant.

## CONCLUSIONS

Not all strains of the pea-nodule organism are identical in their ability to aid the host plant. One strain was found which produced nodules on all five of the host species studied, but which did not benefit the plant.

A strain specificity has also been demonstrated. A culture originally isolated from broad bean (*Vicia faba*) produced better results on *V. faba* than did a culture originally isolated from peas. The pea culture, on the other hand, gave much better results on both peas and sweet peas than did the *V. faba* culture.

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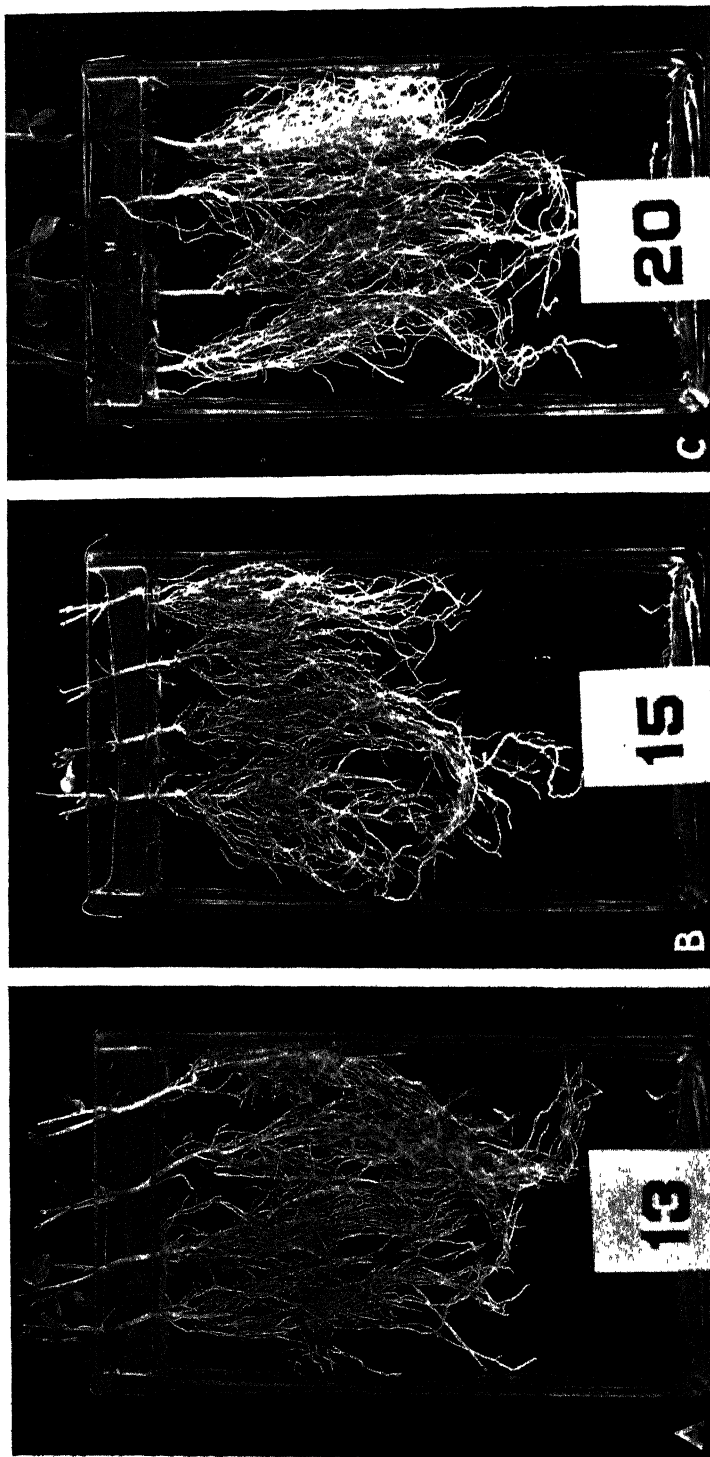


FIG. 6.—Roots of sweet peas (*Lathyrus odoratus*) showing type of nodule formation: A, Inoculated with strain 13. Several medium-sized nodules, scattered over the entire root system. B, Inoculated with strain 15. Numerous small nodules, scattered over the entire root system. C, Inoculated with strain 20. Relatively few large nodules, placed on the upper parts of the root system.

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## STRAWBERRY XANTHOSIS (YELLOW), A NEW INSECT-BORNE DISEASE<sup>1</sup>

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### INTRODUCTION

Strawberries in California are relatively free from the common diseases of this important small-fruit plant. This is undoubtedly due to the climatic peculiarities of California, and especially to its dry, rainless summers. Because of the low atmospheric humidity during the growing season, powdery mildew and the various fungous leaf spots which are so destructive in humid strawberry-growing sections are of very little importance in California. Also, the damage to the berries from fruit-rotting organisms ordinarily is relatively negligible, except in years of abnormally late spring rains.

By far the most important disease of the strawberry in California is xanthosis, or yellows, which forms the subject of the investigations here reported. In the central California coastal region this disease is so destructive as to be the limiting factor in strawberry growing. The productiveness of the strawberry plantations of this region has been on the decline for a number of years. The length of life of the plantings has also decreased. While formerly it was customary to crop strawberry plantings for 4 years, and 5 and even 6 year old patches were not uncommon, now 3 years is the normal life of strawberry plantations in this region, 4 or 5 year old plantings being very rare. So, during the last 8 to 10 years, the average life of a strawberry plantation in this locality has been reduced by at least 1 year. The quality of the fruit also has become inferior. The cause of this general decline has remained obscure. Red-spider or insect injury, excessive irrigation, or not enough, soil conditions such as alkali, lack of drainage, etc., and variety degeneration, all have been advanced by different growers as possible causes for this decline. Horne (?)<sup>3</sup> was the first investigator to recognize the fact that a definite and specific disease was involved and to give a pathological description of it. Just where and when the disease originated is not known. It was first reported from the Watsonville district in 1915

<sup>1</sup> Received for publication Aug. 13, 1927; issued January, 1928. This paper is a revised form of a thesis presented by the writer to the faculty of the University of California in partial fulfillment of the requirements for the degree of doctor of philosophy. Published with the approval of the Director of the California Agricultural Experiment Station.

<sup>2</sup> Grateful acknowledgment is made to R. E. Smith, under whose direction these studies were carried out, for valuable advice and assistance; to W. T. Horne for his sympathetic interest and helpful suggestions; to T. E. Rawlins for advice and assistance in connection with the cytological phase of the work; and to H. H. P. Severin for directing the experiments which established the nonrelation between the causal agents of the strawberry xanthosis and the sugar beet curlytop, and for many helpful suggestions.

<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 1090.

by H. A. Hyde, who sent specimens of affected plants to William T. Horne. It was in the hope of obtaining some information concerning the nature of the disease that the present investigation was undertaken in the spring of 1924 at the suggestion of Ralph E. Smith.

#### NAME OF THE DISEASE

In a preliminary report (12) the term "yellows" was tentatively applied to this disease. This name is unsatisfactory, however, because it is not specific. A great number of plant diseases are designated by the term "yellows," some of which, like aster yellows and peach yellows, belong to the same class (virus) of plant diseases as the one under consideration, while others (cabbage yellows, for example) are caused by well-known fungous parasites. For these reasons it was decided to name this new disease of the strawberry "xanthosis," the Greek equivalent of yellows.

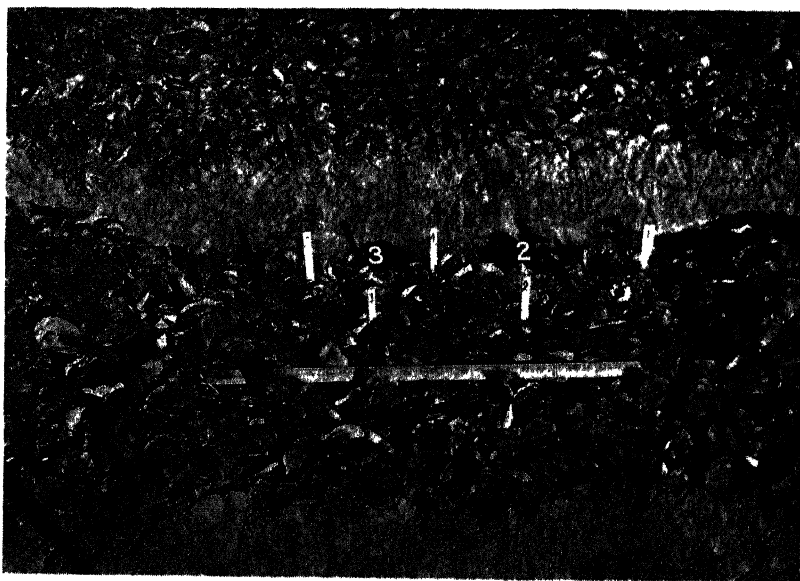


FIG. 1.—Photograph showing the systemic nature of xanthosis. The mother plant (1) is diseased, and so are the four young runner plants (2, 3, 4, and 5) that have come from it.

#### DESCRIPTION OF THE DISEASE

The most conspicuous external symptoms (pl. 1) of xanthosis are: (1) A characteristic crinkling and curling, and usually upward cupping of the leaves. (2) Yellowing of the leaves around the margin and between the larger veins. (3) Dwarfing of the leaves, both petioles and blades. (4) Marked stunting of the growth of the entire plant. (5) Premature reddening, or autumnal color of the older outer leaves. (This is not a very definite symptom, because the leaves of healthy plants also assume a similar color with age or when affected by frost. However, the leaves of diseased plants turn color earlier than those of healthy plants of the same age.) (6) The disease is transmitted through the runners; i. e., every daughter plant arising from a runner from a diseased mother plant becomes diseased. (Figs. 1 and 2.)



Photograph of a young Banner strawberry showing typical xanthosis symptoms. Note the small, crinkled, yellow crinkled leaves.



(7) The first externally visible symptoms manifest themselves in the aerial part of the affected plant; the root system appears per-

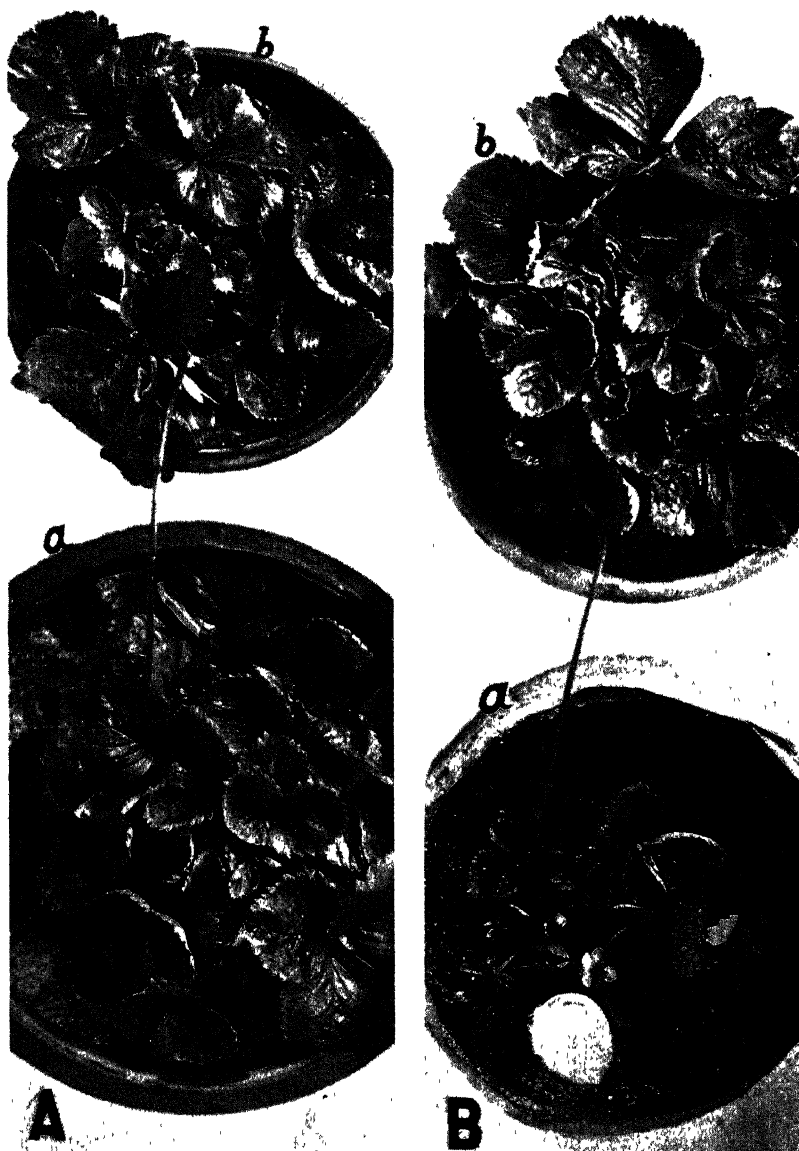


FIG. 2.—Photographs showing both the systemic character of xanthosis and the fact that it is not caused by a soil-inhabiting pathogene. A, Plants in sterilized greenhouse soil; B, sand cultures; a, first-generation runners from diseased Banner plant; b, second-generation runners. The daughter plants remain diseased even when grown on sterilized soil or sand cultures

fectly healthy during the early stages of the disease. (Fig. 3.) Later on, root decay may set in. (8) The affected plants do not die,

but they never recover, remaining permanently stunted.<sup>4</sup> (9) Leaves that are fully grown at the time of infection remain healthy. Only the very young leaves and those developing after the plant has become infected show the chlorotic and distorted symptoms characteristic of



FIG. 3.—Young typically diseased Banner plant, with normal and well-developed root system, illustrating the fact that the top of the plant first shows the disease symptoms

the disease. (10) The fruit is not directly affected; but because of the reduced vitality of the plant and the smaller leaf area, the fruit is smaller in size and hence is considered inferior in quality.

<sup>4</sup> Dying of individual vines or of several plants in spots in the field is quite a common occurrence, and this dying has been attributed by many growers to the disease under consideration. Although the writer does not know the cause or causes of this dying of strawberry plants, he is satisfied that it is not a phase of the disease under discussion, since it occurs also in localities where the disease has not been found.

The symptoms as described apply to what may be termed "typical" specimens. By this is meant infected Marshall and Marshall-like<sup>5</sup> varieties of strawberries grown under field conditions in the central California coastal region. This definition becomes necessary because under certain conditions the symptoms may become partly or wholly masked. Thus, it was found that if typically diseased plants are grown in the greenhouse under cages at 70° to 90° F., the distortion and curling of the leaves usually disappear, although the other symptoms, stunted growth and a chlorotic condition, continue to persist. Varieties which show a certain degree of resistance to the disease do not exhibit all the typical symptoms even when grown side by side with susceptible varieties.

### GEOGRAPHICAL DISTRIBUTION

The disease, while most widespread and most destructive in the central coast district of California, is by no means limited to this region. During the summer of 1925 a careful survey of the strawberry-growing sections of California, Oregon, and Washington was made. In California the disease was not found south of the Salinas Valley on the coast or south of Fresno in the interior. It was found, however, to a limited extent in the San Joaquin and Sacramento Valleys, in Placer County, and in the Sacramento River canyon—in fact, wherever the Banner or Oregon varieties of strawberries are grown. It was also found in Oregon and Washington. Whether it occurs in other parts of the country is not known. It has never been reported from any of the other strawberry-growing States. In England there are apparently several obscure strawberry diseases, variously described by different investigators as "red plant," "cauliflower," "patch," and "little leaf." While most of the English investigators, Johns (8), Lees and Staniland (10), and Ballard and Peren (2), are apparently satisfied that these different terms are synonyms for the same disease, caused by the eelworm *Aphelenchus fragariae* Ritz., others, Turner (17) and Beaumont (3), entertain doubts as to the cause and suggest the possibility of their being diseases of the virus type.

### EXTENT OF DAMAGE

It is not easy to determine the extent of the losses caused by xanthosis, as accurate statistics are lacking. In the hot, interior-valley strawberry-growing sections the damage is relatively small, for under these conditions the infection does not spread readily and the infected plants do not degenerate very rapidly. In the central coastal region, however, the losses are tremendous. An average estimate of a 50 per cent decrease in productiveness of the strawberry plantings in this region is, perhaps, not at all exaggerated,

<sup>5</sup> The Marshall is, of course, a well-recognized horticultural variety (6), which has been under cultivation since 1890. The Oregon, too, has been recognized as a distinct variety (2). In California one of the chief commercially grown strawberries has also been accepted as a distinct variety under the name "Banner." These three varieties are so similar, both in their vegetative growth and in the appearance of their fruit, that there is a question in the minds of many horticulturists and practical growers whether they are in reality distinct varieties. In addition, as if to make the confusion more complete, several local names—Oregon Improved, New Oregon, New Oregon Improved, Oregon Plum, Rose, and Tuttle—have been given to these varieties, or to what are claimed to be improved strains of these varieties. So the term "Marshall-like" or "Marshall type" is used in this paper to cover all these closely related varieties or forms.

considering the threefold nature of the damage. First, there is a decrease in the total yield of fruit, due to the stunting and decreased vitality of the plants. Second, because of this decreased vitality and smaller leaf area the fruit is generally smaller in size and therefore inferior in selling value. Third, the life of the plantation becomes shorter. Not infrequently young plantings in the vicinity of old, infected fields become so badly diseased the first year, because of infection during the early age of the plants, that they are plowed up the second year. Such plantings are a total loss, since no fruit is produced the first year of planting.

### ETIOLOGY

Since the disease was apparently new, and search in the pathological literature of the strawberry failed to give any idea as to its cause, it was necessary to investigate a number of factors as possible causes in the hope that, by the process of testing and eliminating the pathogenic factor might be discovered. The following hypotheses were considered:

1. That the disease is a direct injury caused by the red spider (*Tetranychus telarius* Linn.)

2. That it is caused by adverse soil conditions, especially by the accumulation of salts in such quantities as to produce "alkali" conditions.

3. That it is caused by some soil-inhabiting organism.

4. That it is caused by an infective virus.

5. That it is caused by what has been termed "lack of periodicity." This last hypothesis was early rejected as untenable, however, since it was observed that it was not necessary for plants to overwinter under the mild California climate in order to become diseased. In the spring of 1925 plants that had overwintered in Michigan, where they certainly had become dormant, as well as plants that had overwintered in Oregon, developed the xanthosis symptoms the first season, when planted in Berkeley in the vicinity of infected plants, just as readily as those that had been growing in California continuously.

In the hope of obtaining a lead for possible lines of attack of this problem, the following preliminary experiments were carried out.

### EXPERIMENT 1

From a 2 year-old patch in Mayfield, which showed a high percentage of infection, typically diseased as well as apparently healthy plants of the Banner variety were removed very carefully so as to disturb the root system as little as possible, and transplanted in 7-inch pots as follows:

1. Fifteen diseased plants on soil taken from the field in which they had been growing. Untreated.

2. Same as 1, but the tops of the plants were first dipped in a light oil (Volck) emulsion to kill the red spiders with which the leaves were infested.

3. Fifteen plants on good greenhouse soil.

4. Fifteen apparently healthy plants on the same soil as 1.

5. Fifteen apparently healthy plants on same soil as 3.

The experiment was started on May 20, 1924, and the plants were discarded on July 10, 1925.

No difference was observed during the course of the experiment in the appearance of the originally diseased plants, either between those of lots 1 and 3 (growing on different kinds of soil) or between lots 1, and 2 (dipped and not dipped). Furthermore, the disease gradually developed in the originally normal-appearing plants of both lots 4 and 5, until by the end of August, 1924—i. e., about two months after transplanting—every plant in these two lots had developed typical yellows symptoms.

#### EXPERIMENT 2

Healthy young Banner plants (first-year runners) were planted in 7-inch pots as follows:

1. Twenty-five plants on soil taken from a diseased patch at San Lorenzo. Untreated.
2. Twenty-five plants on same soil as 1 but after it had been disinfected by thoroughly soaking with formaldehyde solution (1 pound in 4 gallons of water) two weeks before planting.
3. Twenty-five plants on rich greenhouse soil. Untreated.
4. Twenty-five plants on the same soil as 3 which had been disinfected in like manner as 2.

The pots were put in saucers, watered carefully so as to avoid leaching the soil and placed outdoors in the plant pathology garden in Berkeley. There were diseased plants in the vicinity (about 25 feet away), and no effort was made to protect the healthy ones from insects. The experiment was started on July 15, 1924, and the plants discarded 13 months later.

It was thought that if the causal agent was a soil-inhabiting organism, the plants of lot 1 would contract the disease, since this soil came from a heavily infected patch and was not treated in any way, while those of lot 2, grown on disinfected soil, would remain healthy. Furthermore, if the causal agent was not a living organism but some physical or chemical peculiarity of the soil, the plants of lot 3, which were grown on a different kind of soil (rich greenhouse soil on which no strawberries had ever been grown), would not develop the disease. Lot 4 was used as a check to test any possible effect of the disinfectant on the soil.

The plants in all four lots grew vigorously, producing runners profusely. By the end of September of the first season four plants in lot 2 began to show signs of decline, but none developed typical xanthosis symptoms. All those of the other lots appeared healthy. By the end of May of the following year—i. e., about 10 months after planting—the condition of the plants was as shown in Table 1.

TABLE 1.—*Number of plants of the Banner variety used in experiment 2, treatment of each lot, and number of plants in each lot which showed typical xanthosis symptoms 10 months after planting*

Lot No.	Treatment	Number of plants used in experiments	Number of xanthotic plants
1	Planted on undisinfected soil from heavily infected patch.....	25	4
2	Same soil as in 1, but disinfected with formaldehyde.....	25	10
3	Planted on rich greenhouse soil; untreated.....	25	7
4	Same soil as in 3, but disinfected with formaldehyde.....	25	5

By the time the experiment was discontinued (August 10, 1925) practically every plant in each lot was showing symptoms of disease. The evidence from these two preliminary experiments, while not final or conclusive, appeared to indicate that xanthosis was caused neither by soil conditions (physical or chemical) nor by a soil-inhabiting organism, nor was it a manifestation of red-spider injury. It was observed that lots 2 and 3, which were situated nearest to a patch of diseased Banner plants, were the first to show yellows symptoms. From this, and also from the general symptomatology of xanthosis, as well as from field observations, the writer was led to suspect that perhaps he was dealing with a disease of the virus type which was transmitted by insects. Subsequent experiments confirmed this suspicion.

Experiments were also carried out to test the three other suspected causative factors, namely, red spiders, adverse soil conditions, and a soil-inhabiting pathogen. Since all these tests gave negative results, and since the evidence establishing the virus nature of xanthosis is, in the writer's estimation, definite and conclusive, it is not considered necessary to give here a detailed description of these experiments. The negative evidence is therefore presented here in a brief form.

#### EVIDENCE THAT XANTHOSIS IS NOT CAUSED BY THE RED SPIDER

Xanthosis symptoms are distinctly different from those of red-spider injury. Red-spider-affected leaves show irregular, characteristically discolored areas, chiefly on the lower surfaces, produced by the chafing mouth parts of the mites, and gradually turn a brownish color and dry up because of loss of moisture through the injured epidermis, but they never develop the marginal chlorosis, the severe stunting, or the crinkling and cupping which are typical characteristics of xanthosis.

Field observations do not show any correlation between red-spider infestation and prevalence of xanthosis. Healthy plants can be found in the field heavily infested with red spiders; and, conversely, typically xanthotic plants can be found practically free from them.

Diseased plants when kept free from red spiders by spraying frequently with light oil (Volck) emulsion did not recover.

Healthy Marshall plants in cages were heavily infested, artificially, with red spiders. During the four and one-half months that these plants were kept under observation they developed none of the xanthosis symptoms.

#### EVIDENCE THAT XANTHOSIS IS NOT CAUSED BY SOIL CONDITIONS

In California strawberries are grown only under irrigation. For the most part the matted-row system of planting is used. The vines are planted on ridges about 2 feet wide and about 8 inches high. The mother plants are set in two parallel rows at the edges of these ridges, about 18 inches apart, and the runners are allowed to root very close, 6 to 8 inches apart, so that the entire ridge is occupied by plants, forming a rather dense, matted row. (Fig. 4.) The plants are irrigated by completely filling with water the ditches between the rows. This is done once a week, and sometimes oftener during the

summer. For the central California coast district, where the soil is usually heavy, this method of culture is, perhaps, the most unfavorable one that could be devised. As no provision is made for drainage, the water stands in the ditches until it evaporates, very little being lost through percolation. Cultivation after each watering is practiced usually only during the first season but not during subsequent years, as it interferes with the picking of the fruit. As the fruit pickers walk in the ditches, the soil there becomes badly packed, making for very unfavorable drainage and aeration conditions. A system of culture of this sort naturally favors the accumulation of salts, especially if the irrigation water happens to be saline, and this is often the case. In view of this situation it was at first thought that the disease might be due to these adverse cultural practices, resulting in an unfavorable soil reaction; too high a concentration of some particular injurious substance, like  $\text{Na}_2\text{CO}_3$ , or in a too high



FIG. 4. —A well-cared-for first-year planting, showing the matted-row type of strawberry culture in vogue in California. At the time this picture was taken, in early summer, this patch showed about 10 per cent xanthosis infection; by fall practically every plant had become diseased.

total concentration of salts. That a similar view was held by others is seen from a brief popular article (1) in which it is stated, more or less as a fact, that this trouble is due to excessive concentration of salts, and the belief is expressed that rainfall may solve the trouble by washing out the salts. Accordingly, during the early summer of 1924, soon after this study was undertaken, representative samples of irrigation water and of soil from healthy and diseased patches were collected and analyzed in an attempt to determine whether or not there was any direct correlation between the reaction of the soil solution and the disease, or between the concentration of a particular salt, or the total concentration of salts in the soil and the disease. Only the ions of the salts that are likely to accumulate in quantities large enough to produce alkali conditions—i. e., Ca, Mg, Na,  $\text{HCO}_3$ ,  $\text{SO}_4$ , and Cl—were determined.

The results of these analyses failed to show any definite positive correlation between the occurrence of xanthosis and either the reac-

tion ( $P_H$ ) of the soil solution or the accumulation of salts in the soil. It is true that, for any one particular field, soil samples from spots containing badly xanthotic plants usually showed a higher total concentration of salts than the samples from spots having healthy or only slightly affected plants, but this relation does not hold true when samples from different fields are compared. For example, soil samples from a patch having typically xanthotic plants showed a total salt concentration of 346 parts per million, while samples from another field having healthy plants showed a total salt concentration of 1,200 to 1,400 parts per million.

Futhermore, it was observed soon after the analytical studies were undertaken that xanthosis occurs on soils in which alkali conditions can not possibly exist. For example, it was found on Banner plants in a garden in the Santa Cruz mountains where the soil was very well drained and the irrigation water came from a mountain spring. The disease was also found in Oregon and Washington, in localities where strawberries are grown without irrigation. Even plants that were grown on light greenhouse soil in pots contracted the disease, as already reported in this paper.

It seems probable, however, that alkali conditions may play a contributory rôle by accentuating the symptoms of the disease. The xanthosis symptoms, like those of other diseases of the virus type, are often masked, partially or completely, and become more prominently manifested whenever the normal growth of the plant is interfered with. For example, there were seven apparently normal plants (variety Nick Ohmer, which exhibits a certain degree of resistance to yellows) growing in pots, adjacent to typically diseased Banner plants. As these were not protected from insects, they had every chance to contract the disease, yet they continued to appear perfectly normal for over a year. At the end of this period they were removed from the pots and transplanted to the garden. Within two weeks after transplanting they began to show the yellows symptoms, and have become and remained typically diseased since. Apparently, although these plants were harboring the infective principle, the degree of resistance that this variety shows was sufficient to prevent the symptoms from manifesting themselves as long as the growth of the plants was not interfered with. But when the growth was checked by the injury to the root system occasioned by the transplanting the symptoms immediately developed. Many other similar instances were observed. The prominence and destructiveness of yellows in the central coast district may be due in part to the accumulation of salts, which is favored by the heavy texture of the soils, the salinity of some of the irrigation waters, and the cultural practices in vogue in this region. While this mild alkali condition may not be sufficiently toxic to cause noticeable injury to normal plants, it accentuates the decline of plants that harbor the infective principle.

#### EVIDENCE THAT XANTHOSIS IS NOT CAUSED BY A SOIL ORGANISM

From the behavior of the disease in the field as well as from the results of the preliminary experiments previously recorded, it appeared unlikely that yellows was caused by a soil organism. The disease spreads with extreme rapidity over the entire field, and not gradually in spots as is the case in most of the plant diseases caused by soil-

inhabiting, root-invading parasites. Furthermore, as stated earlier, the disease manifests itself first in the top of the plant, the root system appearing perfectly normal for some time after the top has become diseased. (Fig. 3.) However, this phase of the problem was further studied from different angles, viz:

1. The root flora. Specimens of healthy and of diseased plants were collected from eight different localities in the State, and their underground parts—roots and crowns—were plated, and pure cultures of the organisms developing on the plates were obtained. In the preparation of the media, the plating of the material, and the isolation of the organisms the usual laboratory technic was followed. The specimens included healthy and diseased plants of all ages, from very old plants with most of their root system black and decaying, to very young runners that had just taken root.

The young, sound roots, whether from healthy or from diseased plants, almost invariably gave sterile plates. Over 150 isolations of fungi and bacteria were made from the older roots, or from partly decayed roots and crowns. No attempt was made to classify the bacteria, or to determine the species of the fungus isolations. The following genera are represented by the fungus cultures: *Alternaria*, *Botrytis*, *Cephalothecium*, *Cladosporium*, *Diplodia*, *Fusarium*, *Oospora*, *Penicillium*, *Phoma*, *Rhizoctonia* and *Verticillium*. In addition, several other isolations produced no spores, and have not been determined as to genus. As determined by preliminary examinations, 42 of the isolations belong to the genus *Fusarium*, and at least 18 distinct forms of this genus are here represented. The flora isolated from healthy plants does not differ from that isolated from diseased ones. In these culture studies no one particular organism was found constantly associated with the xanthosis disease.

2. Runners from healthy plants were allowed to root in pots on soil which had been heavily inoculated with soil from around the roots of typically xanthotic plants. Many dead and decaying roots were also included in the inoculum. The plants remained healthy during the 11 months that they were under observation.

3. A set of 10 runners from xanthotic Banner plants were allowed to root on autoclaved greenhouse soil in 7-inch pots (fig. 2, A), and a second set of similar runners were allowed to root on sterilized sand cultures (fig. 2, B) with Hoagland's solution.<sup>6</sup> After the runners had become established they were detached from the mother plants. Then the secondary runners produced by the daughter plants thus established were allowed to root in sterilized soil as before. This process was carried on in some cases for three generations of runners. Figure 2 illustrates this procedure, *a* showing the first generation runners that came from the original diseased mother plants, and *b* the second generation daughter plants. All show the xanthosis symptoms quite clearly.

All the runners, without a single exception, both on soil and on sand cultures, developed into typically yellowed plants, like the mothers from which they originated, and the plants coming from subsequent generations of runners were also diseased. This is felt to be conclusive evidence that a soil organism is not involved. It is

<sup>6</sup> Hoagland's solution has the following composition: Ca, 159 parts per million; Mg, 54 parts per million; K, 193 parts per million;  $\text{SO}_4$ , 217 parts per million;  $\text{P O}_4$ , 145 parts per million;  $\text{NO}_3$ , 717 parts per million.

true that, notwithstanding all precautions, neither the soil nor the sand cultures could have remained absolutely sterile, yet it is highly improbable that every pot of soil and every sand culture became contaminated with the possible pathogenic organism.

4. Extensive cytological and histological studies failed to reveal the presence of any organism in the tissues of diseased plants. What appears to be a mycorrhizal fungus was often found in the fibrous roots of both healthy and diseased plants. This fungus apparently has no causal relation to xanthosis, for it is equally common in the the roots of healthy and diseased plants.

#### EVIDENCE THAT XANTHOSIS IS NOT TRANSMITTED THROUGH THE SEED

It has been found that xanthosis is not transmitted through strawberry seed. Horne (data unpublished) grew a large number of seedlings from seed of infected plants, and all of these remained healthy as long as they were kept away from infective insects. The writer also grew to maturity over 2,000 seedlings from seed of typically xanthotic Banner plants and all proved to be healthy. The non-transmissibility of xanthosis through the seed is a very important fact, since it offers an easy and certain method of obtaining healthy plants of a variety which has become hopelessly infected. It is true, of course, that strawberries do not breed true; but, by growing large numbers of seedlings, some will be found that approach very closely the characteristics of the mother variety.

#### EVIDENCE THAT XANTHOSIS IS A VIRUS DISEASE

##### TRANSMISSION EXPERIMENTS

##### INSECT INOCULATION

##### EXPERIMENT 1

Forty healthy young Banner plants in pots of steam-sterilized greenhouse soil were divided into 5 lots of 8 plants each and treated as follows: Lot 1 was placed in an isolated spot, away from any other strawberry plants, to be used as a control. Lot 2 was placed in another isolated spot and lightly infested with red spiders taken from leaves of diseased plants. Similarly, the plants of lot 3 were isolated and infested each with 10 strawberry aphids (*Myzus fragaefolii* (Kll.) taken from leaves of diseased plants. The pots of lot 4 were placed in a patch of diseased Banner plants and left unprotected from insects. The diseased plants in this patch were heavily infested with aphids. Other insects, as well as red spiders, were found on them. The plants of lot 5 were placed in insect-proof cages in the vicinity of the same patch. The experiment was started in the middle of July, 1925.

Lot 1 (the control) remained healthy. These plants became infested with aphids, white flies, red spiders, etc., but apparently these had not come in contact with diseased plants and therefore were not infective.<sup>7</sup> Lots 3 and 4 (i. e., those infested with infective aphids and those left exposed to infective insects, respectively)

<sup>7</sup> The term "infective" is used for the sake of convenience to denote all insects that are known to have fed upon diseased plants, and not merely those that have been proved by tests to carry the infective principle.

began to show xanthosis symptoms about one month after inoculation and developed all the typical symptoms of the disease after another month.

Six of the eight plants that were placed in insect-proof cages (lot 5) failed to grow (a severe treatment with nicotine-sulphate oil emulsion at the time of planting proved too strong for them), but the two that survived developed normally and remained healthy.

One month after inoculation three of the eight plants of lot 2 (those infested with red spider) showed symptoms of the disease; after another five weeks, all were diseased. Aphids appeared on all the plants of this lot soon after the first three plants had begun to develop yellows symptoms, and it seems probable that these aphids inoculated the remaining five plants from the first three. It was at first thought that the red spider transmitted the virus to the three plants which first became diseased. Since, however, in all subsequent experiments the red spider consistently failed to transmit the disease, it is now believed that such was not the case. In October of the same year, a careful inspection was made of the nursery from which the plants were received, and a small amount of xanthosis infection was found there; it is therefore possible that these three plants of lot 2 were actually harboring the infective principle even though they did not show symptoms of the disease at that time.

After this experience, it seemed no longer possible to trust Banner variety plants grown in California or Oregon nurseries for experimental purposes, even when they appeared perfectly healthy. In all subsequent transmission experiments Marshall plants from the Eastern States, where the xanthosis disease has never been reported were employed, or, better still, Banner seedlings that had been kept under insect-proof cages from the time of the germination of the seed.

#### EXPERIMENT 2

During the summer of 1926, essentially the same experiment as that recorded above was repeated, with the following modifications: (1) Healthy Marshall plants from Michigan instead of Banner were used; (2) an additional control was provided by a lot of eight plants which were infested with 10 noninfective aphids;<sup>8</sup> (3) the aphids, both infective and noninfective, were allowed to feed on the plants for 10 days, after which they were killed by dusting the plants with a dust containing 4.5 per cent of nicotine sulphate. This was done in order to differentiate between the direct injury of the insects and the effect of the virus.

The plan of the experiment and the results obtained are summarized in Table 2. The results verify those of the previous year (experiment 1 in that they show the ability of the aphids to transmit the causal agent of xanthosis, but give negative evidence for the red spider. This is true, also, of all subsequent experiments.

<sup>8</sup> It was found by tests that all strawberry aphids do not carry the infective principle. In fact, it is quite easy, apparently, to obtain noninfective aphids, for until they have fed on diseased plants, they are clean. There are in the writer's stock three cultures of noninfective aphids, one obtained from strawberries from a garden in Yuba City, a second from a garden in Oakland, and a third from wild strawberries growing on the sands of the beach in San Francisco. A fourth clean culture, from the greenhouse of W. T. Horne, in Berkeley, was lost through accident.

TABLE 2.—Results of transmission experiment 2 with plants of the Marshall variety from Michigan

[Planted April 26, 1926; infested with insects June, 7, 1926]

Lot No.	Treatment	Number of plants used	Number of plants diseased	Percentage of infection
1	Control 1.—No insects.	8	0	0
2	Control 2.—Plants infested with 10 noninfective aphids.	8	0	0
3	Plants infested each with 10 infective aphids.	8	8	100
4	Plants infested with red spiders (about 20 individuals for each plant) taken from leaves of diseased plants.	8	0	0
5	Pots containing the plants placed in a patch of diseased strawberries and left exposed to insects.	8	8	100
6	Control 3.—Plants placed in same vicinity as lot 5, but protected by insect-proof cages.	8	0	0

## EXPERIMENT 3

In this and all subsequent experiments the plants were carefully guarded against chance infection by being kept in insect-proof cages. The types of cages used are illustrated in Figure 5. For

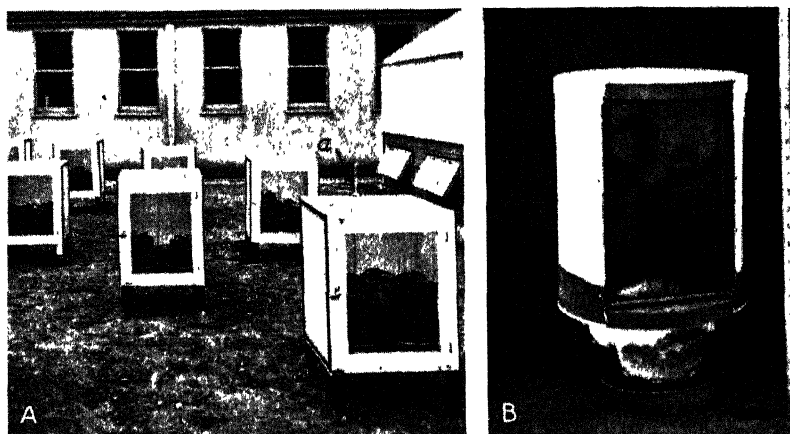


FIG. 5.—Types of insect-proof cages used in transmission experiments: A, large type in which four to six plants may be placed; B, small type for individual plants; a, glass tube for watering plants

the small individual cages (fig. 5, B) India lawn cloth was used. The large ones (fig. 5, A) have solid bottoms, glass tops and doors, and three sides covered with unbleached muslin. In both the large and small cages the pots were placed in saucers, and the plants were subirrigated in order to avoid the necessity of lifting the cages to water the plants, thus eliminating the danger of accidental introduction of insects into the cages. The large cages were supported by legs which either rested in pans containing water with a layer of oil, or were coated with tree tanglefoot, so that no ants could crawl in. The doors of these large cages were seldom opened, the plants being watered through a glass tube (fig. 5, A, a) which was kept plugged with cotton when not in use. Autoclaved<sup>9</sup> greenhouse soil was employed in all these experiments.

<sup>9</sup> The soil was autoclaved in order to kill weed seed and insects and their eggs.

On July 19, 1926, 21 healthy young Banner seedlings, in individual insect-proof cages since germination, were divided into four sets and treated as follows:

1. Six plants infested each with 15 infective aphids.
2. Five plants infested each with 15 noninfective aphids; control.
3. Five plants infested each with about 20 red spiders from leaves of diseased plants.
4. Five plants infested each with about 20 noninfective red spiders.

The plants thus treated were kept in the greenhouse. The temperature range of the greenhouse was not checked at that time, though it was supposed to run from 70° to 90° F. Later, when there was occasion to make an accurate check of the greenhouse temperature, it was found to reach a much lower minimum. (Fig. 11 and Table 11.) The aphids and the red spiders, both infective and noninfective, were killed after they had been allowed to feed for two weeks, by spraying the infested plants with a dilute nicotine-sulphate oil emulsion.

In about three weeks after inoculation, five of the six plants of lot 1 (infective aphids) began to appear stunted and somewhat chlorotic, but they did not show the typical xanthosis symptoms. Those of the other three lots remained normal. In another four weeks the sickly condition of these five plants of lot 1 became accentuated; the difference between these and the plants of the other three lots which still continued normal, was decidedly noticeable. The stunted growth became more prominent, and the new leaves were chlorotic and progressively smaller in size, but there was very little distortion or cupping of the leaves.

Bearing in mind the well-known influence of high temperatures on the manifestation of symptoms in virus diseases of plants, as well as the results of experiment 4, recorded later, it was thought that this might be a masking of the typical yellows symptoms in the inoculated plants due to the relatively high temperature prevailing in the greenhouse. Accordingly, on August 28, all the plants used in this experiment were removed outdoors, but were still kept under the cages. Within a period of 10 days after removal, the five plants of lot 1 had become typically diseased while the rest of the plants remained healthy. So, apparently, the partial masking of the xanthosis symptoms was due to relatively high greenhouse temperature.

The results of experiment 3 are summarized in Table 3.

TABLE 3.—*Results of transmission experiment 3 with Banner seedlings 5 to 6 months old kept under cages from the time of germination*

Lot No.	Treatment	Number of plants used	Number of plants diseased	Percentage of infection
1	Plants infested each with 15 infective aphids.....	6	5	83.3
2	Control, plants infested each with 15 noninfective aphids.....	5	0	0
3	Plants infested each with about 20 "infective" red spiders.....	5	0	0
4	Plants infested each with about 20 noninfective red spiders.....	5	0	0

## EXPERIMENT 4

Seven healthy young plants (runners from Banner seedlings) in individual insect-proof cages were infested each with 25 infective aphids. Six similar plants were infested each with 25 noninfective aphids to act as controls. The plants of both lots were placed on a low bench (about 8 inches from the ground) on the north side of the greenhouse, where it was relatively cool. The temperature of this spot from August 30 to September 13 is shown in Figure 11 and Table 11. The experiment was started July 15, 1926. The aphids, both infective and noninfective, were killed by dusting with nicotine dust after they had fed on the plants for one week.

Two weeks after inoculation, five of the seven plants infested with infective aphids began to show the yellowing and curling character-

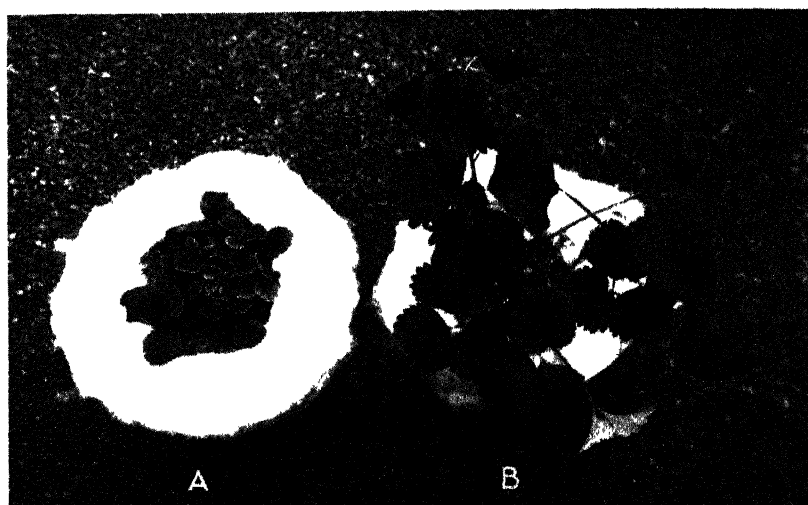


FIG. 6.—A, Banner seedling which became typically xanthotic after being inoculated by means of infective aphids (*Myzus persicae* (Kil.)); B, control; sister plant of A, infested with noninfective aphids.

istics of xanthosis, and within three weeks more they had become typically diseased. (Fig. 6.) The remaining two plants of this lot, as well as those infested with noninfective aphids, remained healthy.

The results of this experiment are summarized in Table 4.

TABLE 4.—Results of transmission experiment 4

Lot No.	Treatment	Number of plants used	Number of plants diseased	Percentage of infection
1	Plants infested each with 25 infective aphids.....	7	5	71.4
2	Plants infested each with 25 noninfective aphids.....	6	0	0

It should be noted that in this experiment the shortest incubation period (time between date of inoculation and appearance of the first symptoms of the disease) of any of the transmission experiments was observed. It was at first thought that mass infection might be a

factor, since in this experiment a rather large number of aphids (25) were used. This, however, was apparently not the case, for later as many as 50 infective aphids to the plant were used without attaining as short an incubation period as that observed in this experiment. Apparently the conditions of this experiment were favorable for an early manifestation of the symptoms, but as these conditions were not controlled, it was not possible to duplicate them.

#### EXPERIMENT 5

In this experiment the large cages (fig. 5, A) were used. These were located outdoors and were protected from the north and west winds by a building and the greenhouse, respectively. One cage, containing four young healthy Marshall plants, was used for each lot. At the time of inoculation, these plants were 1 month old from the time of planting, and had from five to seven leaves, all appearing normal. The experiment was started on May 18, 1926. The five different lots were treated thus:

1. Plants were infested each with five infective aphids.
2. Control; each plant was infested with five noninfective aphids.
3. Plants were infested each with about 20 "infective" red spiders.
4. Control; each plant was infested with about 20 noninfective red spiders.
5. Extra control; plants were kept free from insects.

Results similar to those of experiment 3 were obtained. By the end of the first 25 days after inoculation, the plants of lot 1 (infective aphids) had assumed a sickly appearance (stunted growth and partly chlorotic leaves), but they did not show typical xanthosis symptoms. The controls, as well as the red-spider-infested lots, remained normal.

At this time the plants of lots 1 and 2 were reinfested each with 20 infective and noninfective aphids, respectively. These aphids were killed a week later by dusting with nicotine dust. The red-spider-infested plants were sprayed with 1-100 oil (Volek) emulsion to kill the red spiders.

The sickly condition of the plants of lot 1 became more pronounced during the summer; still, the typical xanthosis symptoms did not develop. The leaves were small and chlorotic around the margins, but there was practically no crinkling of the leaves. The control plants and those infested with red spiders remained healthy.

The temperature of the air inside the big cages was recorded for the two weeks from May 31 to June 13 by placing a thermograph therein, and it was found to be high, reaching a maximum of 98° F. It was thought, therefore, that this apparent partial masking of the symptoms might be due to the high temperature prevailing in the cages. Accordingly, on October 2, two of the four plants of lot 1 were removed from the large cage, covered with small individual cages, and placed in a cooler place (on the same bench as the plants of experiment 4). Within two weeks these plants developed typical xanthosis symptoms. So, in this case also, as in that of experiment 3, the high temperature was apparently the factor which partially masked the symptoms of the disease. Table 5 gives a convenient summary of the results of experiment 5.

TABLE 5.—Results of transmission experiment 5

Lot No.	Treatment	Number of plants used	Number of plants diseased	Percentage of infection
1	Plants infested with infective aphids.....	4	4	100
2	Control plants infested with noninfective aphids.....	4	0	0
3	Plants infested with "infective" red spiders.....	4	0	0
4	Control plants infested with noninfective red spiders.....	4	0	0
5	Extra control plants free from insects.....	4	0	0

## EXPERIMENT 6

This experiment was undertaken to test further whether the red spider may act as a vector for the infectious principle of xanthosis. The plan of the experiment was as follows:

1. Six healthy young plants of the Marshall variety were infested each with 20 to 25 "infective" red spiders, that is with red spiders taken from leaves of diseased plants.

2. Control: Six healthy young plants of the Marshall variety were infested each with 20 to 25 noninfective red spiders.

3. Extra control: Three plants infested each with 10 infective aphids. This additional control was used to determine whether the conditions of the experiment were favorable for the development of the symptoms of the disease.

This experiment was started on September 7, 1926, and was continued for two and a half months. The plants were kept under individual small cages outdoors in the same place as those of experiment 4.

Both sets infested with red spiders remained healthy, while the three plants of the additional control (infective aphids) became diseased. So, from the results of this and previous experiments, it is reasonably safe to state that *Tetranychus telarius* Linn. is not an agent of transmission for the infective principle of xanthosis.

## MECHANICAL INOCULATION

## PRELIMINARY EXPERIMENT

In the summer of 1925 a preliminary experiment was tried to test the transmissibility of xanthosis by inoculating plants with juice extracted from crushed leaves of diseased plants. Eleven apparently healthy Banner plants were inoculated, by means of a hypodermic syringe, with extracted juice from leaves of diseased plants, a second lot of 11 plants were inoculated in a similar manner with juice extracted from leaves of healthy plants, to act as controls, and a third lot of 11 plants were injected with sterile water. The plants used had been in the vicinity of diseased plants for some time and were not protected against insects.

Nine out of 11 of the plants inoculated with diseased juice developed xanthosis symptoms within two months after inoculation, as against 4 out of 11 for each of the two control sets. Although no great importance could be attached to these results, since the plants were left unprotected against infective insects, it was felt that they might

be of some significance. The following two experiments, performed under carefully controlled conditions, gave negative results and failed to verify this supposition.

#### EXPERIMENT 7

The plants used in this experiment were healthy young plants from runners of Banner seedlings, kept under cages since germination. The juice was extracted in the following manner: The leaves were thoroughly macerated in a mortar, a little water was added, and after continuing the maceration a little longer so as to mix the water with the crushed tissue, the liquid was strained through cheesecloth. The highly turbid liquid was allowed to settle for about five minutes, after which the supernatant liquid, still cloudy but free from large particles, was drawn into the barrel of a hypodermic syringe and immediately injected into the leaves, petioles, and crowns of the plants. The juice was not filtered, for it was decided simply to test its infectiousness first, and if the results of the inoculation were positive, then to test the filterability of the infectious principle. In addition to the thorough injection of the juice by means of the hypodermic needle, some of the leaves of the inoculated plants were mutilated by crushing them between the thumb and the forefinger and rubbing on the injured tissue similarly crushed leaves of diseased plants. To make the inoculation still more thorough, small slits were made in the petioles of the leaves and some of the macerated diseased leaf tissue was inserted. Six plants were inoculated in the manner indicated, while a second lot of four plants were similarly treated, but with juice and crushed tissue from healthy leaves to act as controls. The healthy leaves came from Banner seedlings that had been kept under cages since germination. Both sets were placed outdoors, in cages of the type shown in Figure 5, A. The inoculations were made on May 27, 1926, and the experiment was continued throughout the entire summer.

Both the inoculated and the control plants remained healthy.

#### EXPERIMENT 8

Because of the relatively small number of plants used in the preceding experiment, it was decided to repeat it. A lot of nine plants (four Marshall and five Banner seedlings) were divided into two lots, as follows:

1. Five plants, two Marshall and three Banner seedlings, were inoculated with diseased juice and macerated tissue.
2. Control. Four plants, two Marshall and two Banner seedlings, were inoculated with healthy juice and macerated tissue.

The extraction of the juice and the inoculation were done in the same manner as in experiment 7. The plants were covered with the small individual type of cages (fig. 5) and were placed outdoors. The experiment was started on September 9, 1926.

This experiment like the previous one gave negative results. Table 6 summarizes the results of the two experiments.

TABLE 6.—*Results of juice inoculation experiments 7 and 8*

Lot No.	Treatment	Number of plants used	Number of plants diseased
1	Plants thoroughly inoculated with unfiltered juice from diseased leaves, with macerated diseased tissue, and by leaf mutilation	11	0
2	Control: Treated like lot 1, but with juice and tissue from healthy leaves	8	0

Although the number of plants inoculated is not very large, it is believed that the results obtained are sufficient to justify the conclusion that the causal agent of xanthosis is not readily transmissible by direct juice inoculation. It may be possible, of course, to obtain a small amount of infection by this method if a large number of plants are used. Instances are known of typically insect-transmitted virus plant diseases in which a small percentage of infection was secured by means of direct juice inoculation. Severin (14), for example, succeeded, after other workers had failed, in transmitting the curly-top disease of the sugar beet to 9 out of 100 beets inoculated directly with expressed juice from leaves and roots of diseased beets. The fact that xanthosis is not transmitted by direct juice inoculation is not only of theoretical interest, but has a practical significance to strawberry growers. As the roots and tops of the young plants are trimmed before planting, if the disease were easily transmitted by direct mechanical inoculation, there would be danger of spreading the infection from the few diseased plants that are usually contained in each lot to the healthy ones by means of the trimming knives. The results of the two experiments recorded above indicate that such a danger does not exist.

## EXPERIMENT 9

Since the disease was found to be easily transmitted by aphids, but apparently not by direct inoculation with juice extracted from leaves of diseased plants, it was decided to test the infectiousness of juice extracted from infective aphids. Accordingly, about 300 to 400 aphids were taken from leaves of diseased plants, placed in a test tube, and thoroughly crushed by means of a glass rod. About 10 c. c. of water was added, and the mixture was stirred. The crushed bodies of the insects were then allowed to settle to the bottom of the tube, and the supernatant liquid was drawn into the barrel of a syringe fitted to a hypodermic needle, by means of which it was injected into the leaves (midribs and petioles) and into the crowns of six healthy young Marshall plants. The inoculation was done very thoroughly. Small wads of cotton were placed at the opening of the needle wounds and these were soaked with the juice used for inoculation. Four plants were similarly inoculated with juice extracted from noninfective aphids and used as controls. The experiment was started on June 23, 1926, and was continued until November of the same year. The plants were covered with cages of the individual type, kept in the greenhouse for two months after inoculation, and afterwards removed outdoors.

Both the inoculated and the control plants remained healthy. These results can not of course be taken as final, since the number

of plants inoculated was altogether too small. The evidence, however, indicates that the disease can not be readily transmitted by inoculating with juice extracted from infective aphids.

#### SUMMARY OF THE RESULTS OF THE TRANSMISSION EXPERIMENTS

For the sake of convenience, the results of the transmission experiments are summarized in Table 7. The results of experiment 1 are omitted, since, as has been explained, there is some doubt as to whether all the plants used in this experiment were originally free from infection. The results of the preliminary juice-inoculation experiment are also omitted as unreliable, since the plants experimented with were exposed to insect infection.

TABLE 7.—*Summary of the results of transmission experiments 2 to 9*

Treatment	Number of plants inoculated	Number of plants diseased	Percentage of infection
Inoculated with infective aphids.....	28	25	89.3
Control: Inoculated with noninfective aphids.....	23	0	0
Inoculated with "infective" red spiders.....	23	0	0
Control: Inoculated with noninfective red spiders.....	23	0	0
Inoculated with juice extracted from diseased plants.....	11	0	0
Control: Inoculated with juice extracted from healthy plants.....	8	0	0
Inoculated with extract from infective aphids.....	6	0	0
Control: Inoculated with extract from noninfective aphids.....	4	0	0

#### EVIDENCE THAT XANTHOSIS IS NOT CAUSED BY THE SUGAR-BEET CURLY-TOP VIRUS

The question as to whether xanthosis of strawberries and curly-top virus of sugar beets are identical arose, because of the fact that strawberries are grown in the natural and migratory breeding areas of the beet leaf hopper (*Eutettix tenellus* (Baker)) and often in the vicinity of beet fields. Severin (15) has demonstrated that a large number of weeds and cultivated plants of many families are naturally susceptible to curly top and become infected by it. Carsner (4) has shown that weeds and a few crops are susceptible or nonsusceptible to curly top, and he came to the conclusion on circumstantial evidence that the beet leaf hopper may have transmitted curly top to beans in Idaho (5), although he did not see the diseased plants in the field. Recently, McKay and Dykstra (11) arrived at the conclusion, chiefly on circumstantial evidence, that western yellow blight of tomatoes is caused by the virus of sugar-beet curly top. Furthermore, the histological pathology of the strawberry affected with xanthosis is very similar (though not so pronounced), to that of the beet affected with curly top. All the evidence at present available, however, indicates that strawberry xanthosis is a disease distinct from curly top of the sugar beet.

#### INDIRECT EVIDENCE

The symptoms of curly top of the beet are entirely different from those of strawberry xanthosis. It is true, of course, that symptomatology is not an absolute criterion, since the symptoms of a particular disease may vary considerably with different host plants. In the case of the beet curly top, however, there is one symptom—the trans-

parent venation—which is very definite and is invariably exhibited by plants affected with curly top. This feature is entirely absent in the case of xanthosis. Moreover, the beet curly top does not cause very conspicuous yellowing, whereas in the case of strawberry xanthosis, yellowing is the most pronounced symptom. Another piece of circumstantial evidence is found in the fact that the geographical areas where the two diseases are most severe are not identical—in fact they are exactly opposite. In the hot interior valleys of California, where the beet curly top is most severe, strawberry yellows is very mild, whereas in the cool fog belt of the coast the reverse is true.

#### DIRECT EVIDENCE

##### STRAWBERRIES NONSUSCEPTIBLE TO CURLY TOP

Different lots of infective beet leaf hoppers were transferred from curly-top beets to healthy strawberry seedlings and permitted to remain there for a period of about six weeks. Noninfective hoppers were allowed to feed on these strawberry plants and were then transferred to healthy beets, but not a single case of curly top developed. It is evident from this experiment that strawberries are nonsusceptible to curly top.

##### BEET LEAF HOPPERS FAILED TO TRANSMIT STRAWBERRY XANTHOSIS

Different lots of noninfective beet leaf hoppers after feeding on strawberry plants affected with xanthosis were transferred to healthy strawberry seedlings and then to healthy beets, but no disease symptoms developed either in the strawberry seedlings or in the beets. In another experiment, noninfective leaf hoppers after feeding on strawberries affected with xanthosis failed to produce disease symptoms in the beets. It is evident that the beet hopper is not capable of transmitting strawberry xanthosis.

#### INFLUENCE OF TEMPERATURE

That high air temperature exercises a great influence on the symptomatology of virus diseases of plants, tending to obscure, partly or entirely, the symptoms of the disease, is a well-known fact—so well known that it is not necessary here to review the general literature on the subject. Because of lack of appropriate equipment (constant temperature chambers) no absolute figures relative to the influence of temperature on the masking of xanthosis symptoms can be given. The evidence here presented was obtained either from field observations or from a limited number of temperature records.

It was observed soon after these investigations were undertaken and even before evidence was obtained of the infectious nature of the disease, that affected plants, in the field, would make a good growth for three to four weeks in early spring collapse rather suddenly during May, rally considerably during the period from about the middle of June to the end of August, and again collapse during the fall. If a curve were plotted to show this rise and fall in intensity of the disease it would be found practically to coincide with the temperature curves (monthly mean and mean maximum) shown in

Figures 7 and 8, with the exception of the early spring period. The latter discrepancy can be explained by the influence of another factor—the beneficial effect of the dormant period of the winter months.

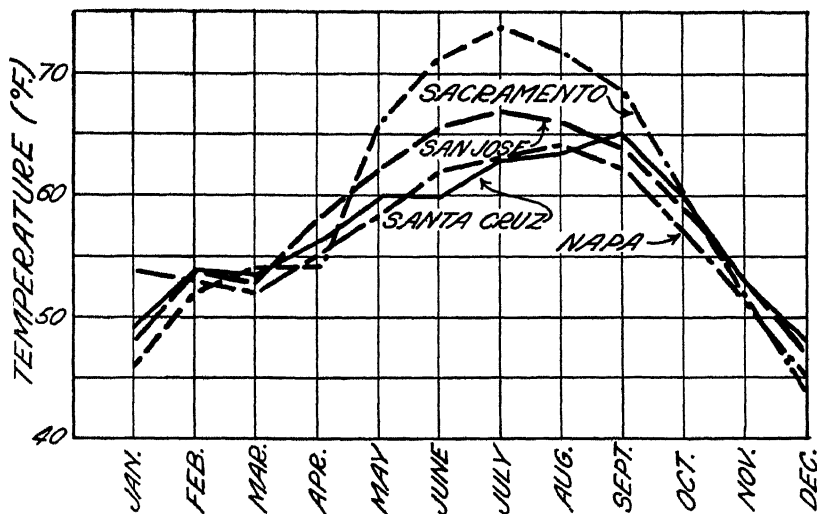


FIG. 7.—Monthly mean temperatures of four different strawberry-growing localities, Santa Cruz, San Jose, Napa, and Sacramento; averages for the two years 1924 and 1925

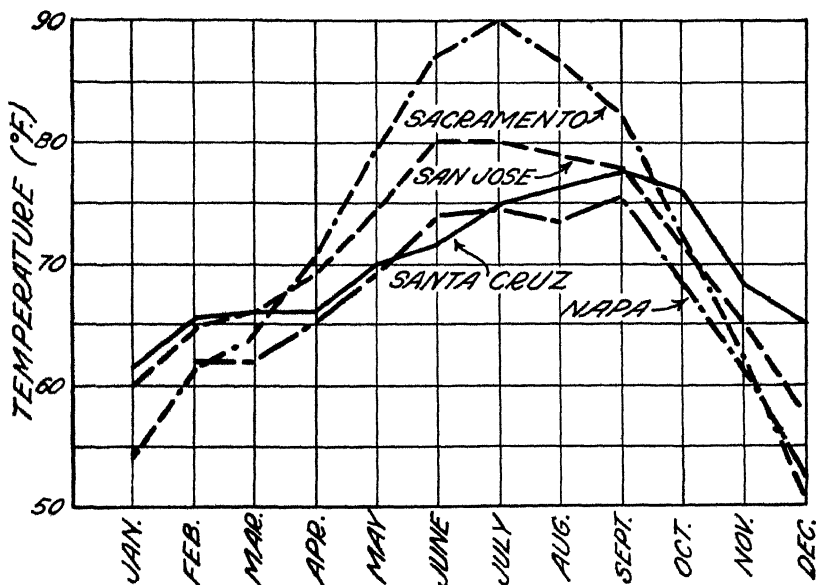


FIG. 8.—Monthly mean maximum temperatures of four different strawberry-growing localities, Santa Cruz, San Jose, Napa, and Sacramento; averages for the two years 1924 and 1925

Horne (7) found that both healthy and sick plants were greatly stimulated by a winter rest period of about two months. It is true, of course, that under the mild climatic conditions existing in Cali-

fornia the plants do not become entirely dormant during the winter, but their growth is markedly checked.

Another significant fact is that while xanthosis occurs in the interior valleys of California, where the temperature is very high during the growing months, it is never serious. The writer had occasion during 1926 to observe a small patch of strawberries at the university farm at Davis. The plants had come from a nursery in which about 10 per cent infection had been found the previous summer. In May this patch was examined and several diseased plants were observed. When examined in August all the plants appeared normal, but on October 24 some showed xanthosis symptoms. Apparently, in this case, the symptoms of the disease were completely masked during the hot summer months. The temperature for Davis is not recorded here, but it is practically the same as that for Sacramento shown in Figures 7 and 8 and in Table 8. The monthly mean and mean maximum temperatures (averages for 1924 and 1925) for four different strawberry-growing localities, Santa Cruz, San Jose, Napa, and Sacramento are also shown. In the first three localities the disease is very prevalent and very severe; in Sacramento it occurs but is not at all serious. As is shown by the curves in Figure 8, the mean maximum temperature of Sacramento for the summer months is 10° F. higher than that of San Jose, and about 15° higher than that of Santa Cruz and Napa for the corresponding months. The monthly mean temperatures (fig. 7) show a similar relation.

TABLE 8.—*Monthly mean and mean maximum temperatures of four strawberry-growing localities in California for 1924 and 1925*

MEAN TEMPERATURE (° F.)												
Locality	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Santa Cruz.....	49.2	54.3	53.4	55.7	59.6	60.1	62.9	63.4	65.3	52.4	52.9	48.2
San Jose.....	48.1	54.0	53.0	57.8	61.9	65.5	67.0	65.9	61.0	59.4	53.4	37.2
Napa.....	53.7	52.8	51.6	55.2	58.4	61.8	63.0	63.0	61.8	57.1	50.7	45.2
Sacramento.....	46.1	52.1	53.6	53.9	60.2	71.5	73.8	71.6	68.7	60.0	52.4	44.1
MEAN MAXIMUM TEMPERATURE (° F.)												
Santa Cruz.....	61.5	65.4	66.1	66.1	70.1	71.5	74.8	75.9	77.5	73.0	68.5	65.2
San Jose.....	59.8	64.4	65.7	69.3	74.3	80.1	80.1	79.0	77.7	71.4	65.2	57.1
Napa.....		61.8	62.2	65.2	69.3	74.1	74.4	73.6	75.6	68.4	61.0	51.8
Sacramento.....	53.7	61.2	63.7	70.5	79.3	87.0	90.0	86.7	82.2	72.0	61.6	49.7

A further illustration of the masking effect of high air temperature is seen in the following example: In the fall of 1925 four typically diseased Banner plants, in 5-inch pots, were placed in an insect-proof cage, similar to those illustrated in Figure 5, A, but provided with double walls of muslin cloth. The following spring, when growth started, one of these plants showed a very slight yellowing of the margins of the leaves, while the other three appeared entirely normal and remained so during the summer. The temperature of the air in this cage was recorded for a two-week period (May 10 to 24) by placing a thermograph in the cage, and the temperature of the outside air for the same period was recorded by means of another thermograph. These records are shown in Figure 9 and Table 9.

It is seen that the temperature of the air inside the cage reached a point as high as 106° F., while that of the outside air hardly rose above 83°. On May 10 two of the normal-appearing plants were removed from the cage and placed outdoors in a spot away from any diseased plants. To serve as controls, two healthy Banner seedlings which had been kept in a cage similar to that of the diseased ones were likewise removed from their cage and placed outdoors in the same locality as the other two. The outdoor temperature for this period ranged from 48° F. to 78° F. The controls remained healthy throughout the summer. The young leaves of the other two plants began to show a marginal yellowing three days after the plants were

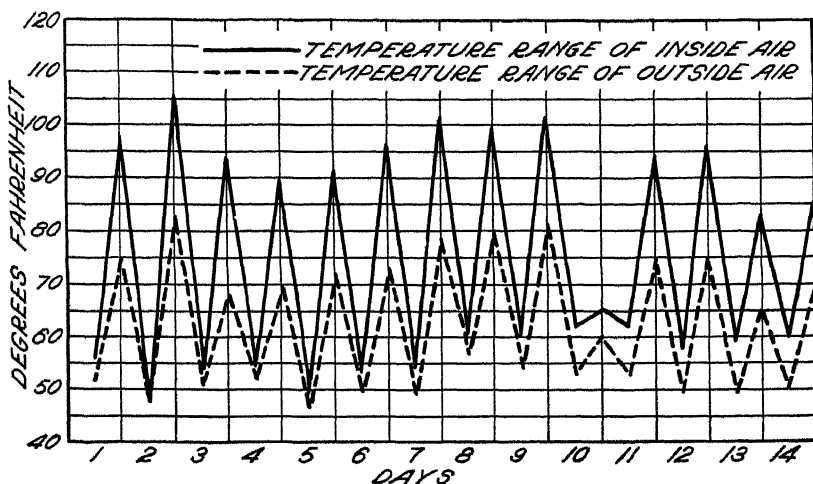


FIG. 9.—Daily range of air temperature inside and outside a double-lined insect-proof cage for the two-week period, May 10-24, 1926

removed outdoors, and all the young leaves subsequently developed exhibited all the typical xanthosis symptoms. So it is apparent that, while the plants continued to harbor the infectious principle, the high temperature within the cage had the effect of masking the symptoms.

TABLE 9.—Minimum, maximum, mean, mean minimum, and mean maximum temperatures of the air inside and outside a double-lined insect-proof cage, for the two-week period May 10-24, 1926

Air	Minimum	Maximum	Mean	Mean minimum	Mean maximum
	° F.	° F.	° F.	° F.	° F.
Outside cage.....	46.0	83.6	61.9	50.1	73.8
Inside cage.....	48.0	106.0	75.5	55.5	95.5

In discussing the results of transmission experiments 3 and 5, it was stated that the disease symptoms of infected plants were partly masked. A comparison of the daily range of temperature inside and outside [the cages used in experiment 5 is shown in Figure 10 and Table 10 and of the inside and outside of the greenhouse (experiment 3) in Figure 11 and Table 11. It is interesting to note that in all these cases where partial or complete masking occurred the

maximum temperature had reached 90° F. or above, and that no masking occurred at a temperature of 84° or below. It is important to consider here that a continuous exposure to high temperature may not be necessary to produce masking effects. Tompkins (16), working with the mosaic of potato, found that "relatively short exposures

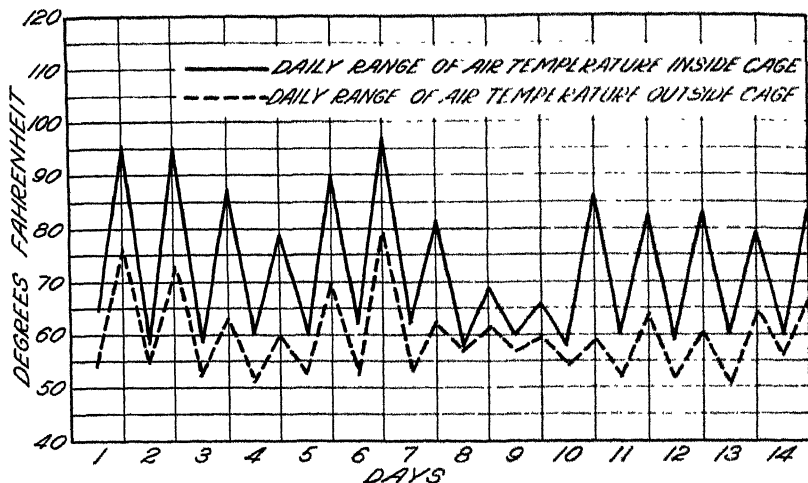


FIG. 10.—Daily range of air temperature inside and outside the large type of insect-proof cages shown in Figure 5, A

to air temperatures above that of the critical air temperature (23° to 24° C.) were sufficient to mask the symptoms of mosaic." Figure 10 and Table 10 illustrate the fact that in studying the masking effect of temperature the maximum rather than the mean should be

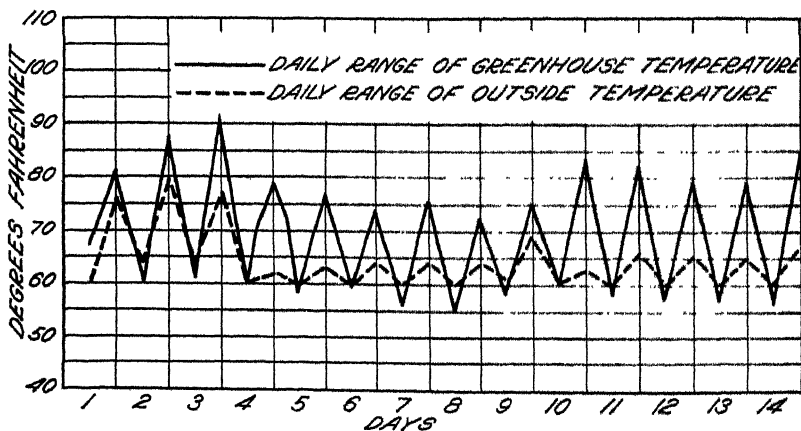


FIG. 11.—Daily range of air temperature inside and outside the greenhouse for the two-week period August 30-September 13, 1926

considered. Thus, for two days, June 8 and 9, the temperature was relatively low (not above 68° F.), so that the mean temperature for the two weeks is relatively low, yet the intermittent exposure to high temperatures was sufficient to partly mask the symptoms. The same fact is illustrated by the curves in Figure 11.

TABLE 10.—*Minimum, maximum, mean, mean minimum, and mean maximum temperatures of the air, inside and outside the large type of insect-proof cages shown in Figure 5, A, for the two-week period May 31–June 14, 1926*

Air	Minimum	Maximum	Mean	Mean minimum	Mean maximum
	° F.	° F.	° F.	° F.	° F.
Outside cage.....	51	79	59.7	53.8	65.7
Inside cage.....	58	98	72.7	59.9	85.5

TABLE 11.—*Minimum, maximum, mean, mean minimum, and mean maximum temperatures of the air inside and outside of the greenhouse for the two-week period August 30–September 13, 1926*

Air	Minimum	Maximum	Mean	Mean minimum	Mean maximum
	° F.	° F.	° F.	° F.	° F.
Inside greenhouse.....	55	91	68.8	58.0	79.6
Outside greenhouse.....	60	80	64.0	60.4	67.6

Although the evidence here presented does not permit the giving of absolute figures for the critical temperature, it seems to indicate that no noticeable masking of xanthosis symptoms occurs at about 80° F. or below, and that above this temperature partial or complete masking takes place.

#### CYTOLOGICAL AND HISTOLOGICAL STUDIES

Extensive cytological and histological studies of the different parts of the plant—root, crown, stem, and leaves (petioles and blades)—were made for the purpose of discovering whether or not any organism or any abnormalities could be found associated with the disease. Over 500 slides of fixed and stained material were examined, in addition to a large number of free-hand preparations made from fresh material. For killing and fixing, the alcohol-formalin-acetic acid solution (100 c. c. of 50 per cent alcohol, 6.5 c. c. of formalin and 2.5 c. c. of glacial acetic acid) was used. For the most part the staining was done with Haidenhain's iron-alum haematoxylin, but Fleming's triple stain was also used. The leaves were found rather unsatisfactory for a critical examination of cell content, since the cells contain plastids and other normal structures which stain heavily and obscure the vision.

#### MYCORRHIZAL FUNGUS IN ROOTS

What appears to be a mycorrhizal fungus has been found almost invariably in the cortex of the fibrous roots, but it has never been observed in the young succulent primary crown roots. This fungus is apparently widely distributed in the soil, for it was found in the roots of plants collected from different fields in widely separated localities in California. Jones (9) lists the strawberry as one of the many plants in the roots of which he found a mycorrhizal fungus. Incidentally, the fungus found by the writer seems to be identical with that described by Jones. No attempt was made to isolate and

culture the fungus. Whether it may be considered as a parasite or as a harmless, perhaps even beneficial, symbiotic organism can not be definitely and accurately determined until it has been obtained in pure cultures. The invaded roots appear perfectly normal externally, and no lesions occur in the tissues in which the fungus is found. Certain it is that this fungus has no casual relation to xanthosis. It occurs as commonly in the roots of healthy plants as in those of the diseased.

#### ABNORMALITIES IN THE LEAVES

The palisade cells of the chlorotic areas of the leaves of diseased plants are noticeably shorter and more compact than those of the leaves of healthy plants, or than those of the green areas of the diseased leaves. This abnormality is apparent both in free-hand sections of fresh material and in fixed and stained preparations.

#### DARK-FIELD STUDIES

Thin free-hand sections of fresh material—leaves (blades and petioles) and roots—of typically xanthotic plants were examined immediately after cutting with a Leitz dark-field microscope. Also drops of juice obtained by gently rolling the midribs, the petioles, and the succulent crown roots between two glass rods until a drop of the liquid oozed out from the cut end, were placed on slides and examined in the same way. No organism of any kind could be seen either in the cells of the sections or in the extracted juice.

#### TISSUE DEGENERATION AND CELL INCLUSIONS

A degeneration of the tissue of the pericycle region of the roots, accompanied by certain conspicuous cell inclusions, was found constantly associated with the disease. These abnormalities were so strikingly similar to those described by Rawlins (13) for sugar beets affected by curly top that the same terminology is, for the sake of convenience, adopted here.<sup>10</sup> The two types of cell inclusions are therefore designated as x and y bodies, respectively.

The tissue degeneration is chiefly found in the pericycle, but it often extends to the adjacent phloem, and more rarely to the endodermal cells. Figure 12, A and B, illustrates this tissue degeneration, shown by the black-staining groups of cells. What is considered to be the first state in the process of degeneration is that certain pericycle cells become filled with what appears to be reticulate cytoplasm (fig. 13, A, *a*), which later becomes granular. About this time the nucleolus has disappeared, and the nucleus shows decided signs of disintegration. The nuclear membrane appears shrunken, inclosing some amorphous granular material, staining black with Haidenhein's haematoxylin and red with the triple stain. In what is considered to be a later stage in the process of degeneration, the nuclear membrane incloses only a small amount of granular material, and it is either shrunken or very well rounded as if filled with liquid (fig. 13, B, *n*); and the part of the cell normally filled with cytoplasm contains either x or y bodies, or both. The x-bodies (fig. 13, A, *x*) are composed of dense, amorphous material which stains black with

<sup>10</sup> In addition to the abstract cited, through the courtesy of its author, the writer was privileged to read the original manuscript of the yet unpublished complete work of T. E. Rawlins.

haematoxylin and red with the triple stain. The y-bodies (fig. 13, B.) are identical in their staining reactions with the x-bodies, but have a more definite shape than the latter, being spherical or nearly spherical, and usually appearing to possess a distinct membrane. Neither the x nor the y bodies show any organization of structure. In what appears to be the final stage in the process of degeneration, the remnant of the nucleus and the x or y bodies disappear, and the entire cell is filled with amorphous, somewhat granular material, staining black with haematoxylin.

Observation of hundreds of sections has led the writer to believe that the process of degeneration is as follows: In what has been considered the first stage the nucleus is practically intact, though showing signs of degeneration; in later stages the nuclear degeneration has proceeded further, until most or all of the nuclear material

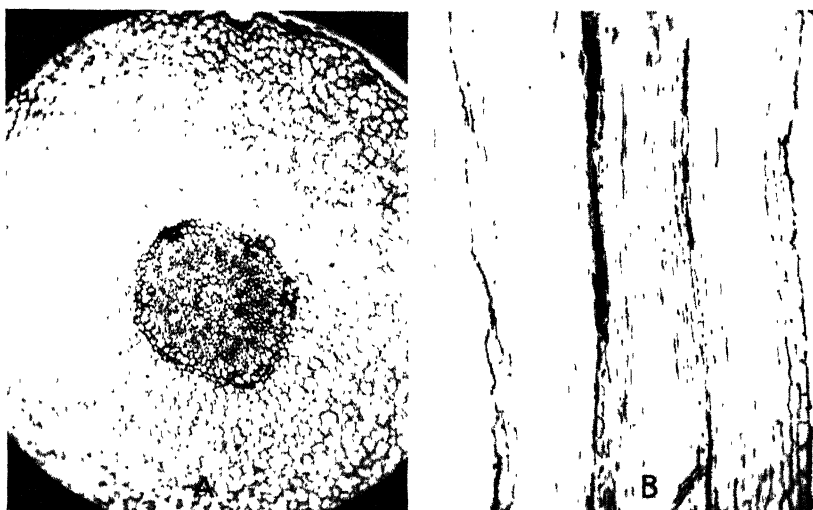


FIG. 12.—A, Cross section of young root of xanthotic Banner strawberry showing degeneration of some pericycle and adjoining cells,  $\times 335$ ; B, longitudinal section of young root of xanthotic Banner strawberry showing tissue degeneration in the pericycle region,  $\times 400$

has disappeared from the nuclear membrane, and at the same time the x or y bodies, or both, have made their appearance. No cells have been found containing x or y bodies in which the nucleus was not degenerated. Finally, both the remnant of the nucleus and the x and y bodies become indistinct.

#### SIGNIFICANCE OF THE ABNORMALITIES

Just what significance may be attached to the abnormalities described, and especially to the peculiar cell inclusions, can not be definitely stated. The fact that these abnormalities have been found constantly associated with the disease, but have never been found in roots of healthy plants, would suggest that they must have some relation to the disease. It is not believed that they bear any causal relation, however. Neither the x nor the y bodies show any structure that would suggest the possibility of their being organisms. They are probably degeneration products of the cell. The fact that



FIG. 13.—A, Photomicrograph of longitudinal section of young root of xanthotic Banner strawberry showing pericycle cells containing x-bodies (x) and a cell filled with reticulate, black-staining cytoplasm (a),  $\times 1570$ ; B, pericycle cell with degenerated nucleus (n) and a typical y-body (y),  $\times 600$ .

they exhibit the same affinity for stains as the nucleus, and that they make their appearance in the cell simultaneously with the partial or complete disappearance of the contents of the nucleus, would suggest that they may be extruded nuclear material.

The same type of pathological histology and the same type of cell inclusions described here were found by Rawlins (13) in sugar beets affected with curlytop. Yet, as is shown earlier in this paper, the evidence indicates that the two diseases, xanthosis of the strawberry and curlytop of the sugar beet, are not identical. If it is correct to consider these cell inclusions as degeneration products, it must be concluded that two distinct, but perhaps similar, causal agents may bring about identical pathological manifestations. Until more is known of the nature of viruses and the mechanism by which they bring about the observed pathological phenomena in plants, any interpretation of the nature of the various abnormalities found must, of necessity, be of a speculative character.

#### SPECIFIC AND VARIETAL RESISTANCE

An extensive project to test varietal resistance in strawberries is now in progress. The results of these tests will not be known for some time; here are given only the results of the observations of the last two years regarding specific and varietal resistance. Of the cultivated varieties, the Marshall type, in which are included the Marshall, Oregon, Oregon Improved, New Oregon, Oregon Plum, and Banner varieties, is the most susceptible to xanthosis. The Nick Ohmer, the Melinda, and the Superb are slightly more resistant than the Marshall type. The Corsican (Uncle Jim) is interesting in that it contracts the disease very readily and shows the yellow leaf very prominently, but does not become greatly stunted. The following varieties have thus far shown a rather high degree of resistance: Early Ozark, Howard 17, Dr. Burrill, Dunlap, Premier, Parsons, Campells Early, August Luther, and Americus. They are not immune, however, for when exposed to infection they contract the disease, but they do not develop all the typical symptoms. The stunting of the plants, for example, is relatively slight compared with that of the plants of the Marshall type. Of the wild species, the beach strawberry, *Fragaria chiloensis* Duch., is immune to the disease as far as one can judge from external symptoms. Whether or not it can harbor the virus has not been determined. The same is true of practically all of Etter's varieties and new selections. Over 70 selections of these Etter strawberries have been grown for a year in the immediate vicinity of diseased Banner plants, under conditions most favorable for infection, and with the exception of two, all have remained healthy. No transmission experiments were tried to determine whether or not the infective principle is harbored by these normal-appearing selections. As all of Etter's varieties contain, to a smaller or greater degree, the *chiloensis* blood, they owe perhaps their high resistance to their wild parent.

A second wild species, the wood strawberry, *Fragaria californica* C. & S., is apparently susceptible. Plants of this species, growing next to a xanthotic Banner plant in a garden in the Santa Cruz Mountains, contracted the disease. This is the only instance in which xanthosis was found on wild strawberries.

## POSSIBLE CONTROL MEASURES

No definite means of control can be given at this time. The work thus far has been chiefly concerned with the etiology of the disease. There are, however, certain methods of attack that readily suggest themselves as control possibilities. From the present limited knowledge of the nature of the causal agent or agents of diseases of the virus type, it is clear that no curative measures can be employed. Preventive methods, however, have been used in controlling diseases of this kind, and in many cases these have been very successful. These methods consist chiefly in the use of planting stock which has been obtained as free from a particular virus disease, or a combination of such diseases, as possible by means of roguing, seed indexing, etc., and in further eliminating the infection by inspecting the plantings several times during the growing season and removing any plant that is found to be diseased.

From observations in the field, as well as from the results of certain experiments which were started last year and are still in progress, it is strongly believed that roguing will prove effective in controlling the xanthosis disease, or at least in greatly checking its spread. There are, however, certain difficulties which militate against an easy application of this method. The most important of these is the fact that, as previously stated, the symptoms of the disease are often completely masked under certain conditions; and, so, in roguing, plants which harbor the virus, but appear healthy, will not be destroyed, and these will serve as a source of infection. The second great difficulty is that even when there is no complete masking the symptoms of yellows are not very sharp and readily defined during the early stages of the disease, and so a certain degree of skill is required to recognize the doubtful cases. But notwithstanding these difficulties it is felt that this method of control is capable of practical application if carried out thoroughly under the supervision of trained inspectors.

Another possible method of control may be suggested here, but what measure of success will attend its use can not even be predicted until more experimental work has been carried out. If it is found that xanthosis is specific as to its vector; that is, if it is transmitted only by the strawberry aphid, it is possible that it might be controlled effectively, indirectly, by spraying or dusting with nicotine, since the aphid is very readily killed by this means.

A more radical means of control lies in the development of resistant varieties. This is a long process, requiring years of breeding and careful selection, but in this case it appears very promising for two reasons: (1) A large number of commercial and semicommercial varieties of strawberries show a marked degree of resistance to xanthosis, and so there is available an enormous amount of valuable material for breeding; (2) the strawberry is an exceedingly convenient plant for breeding, since it is propagated vegetatively. Once the desired cross has been secured, the work is ended; we are not concerned about its genetic constitution, and it is not necessary to continue the breeding until a pure line is obtained, as in the case of seed-propagated plants.

In view of these considerations, an extensive breeding project has been undertaken for the purpose of developing a variety that will

be resistant to xanthosis and that will possess the desirable commercial qualities to meet the agricultural conditions in California.

#### SUMMARY

This paper presents the results of investigations on the etiology and transmission of a new insect-borne disease of the strawberry (*Fragaria*). The term "xanthosis" (the Greek equivalent of "yellows") is proposed as a name for this disease, the symptomatology of which is described.

The economic importance and the geographical distribution of the disease are discussed.

Experimental evidence is given which shows that xanthosis is not due to injury from red spider (*Tetranychus telarius* Linn.), to lack of periodicity, to soil conditions, or to a soil-inhabiting pathogene, but that it is caused by some infectious principle of the virus type. This is the first disease of this nature to be reported on the strawberry.

The disease has been successfully transmitted from sick to healthy plants by means of infective strawberry aphids (*Myzus fragaefolii* Ckll.). Aphids that have not fed on diseased plants are noninfective.

The red spider is not capable of transmitting xanthosis.

Mechanical inoculation with unfiltered juice from diseased plants, or with extract from the crushed bodies of infective aphids, as well as by means of leaf mutilation, failed to transmit the disease.

Xanthosis is not transmitted through the seed.

Circumstantial as well as direct evidence is given to show that xanthosis is not caused by the virus of the sugar-beet curly top. The beet leaf hopper (*Eutettix tenellus* (Baker)) was found incapable of transmitting the causal agent of xanthosis either to strawberries or to beets.

The effect of temperature on the masking of the symptoms of the disease is discussed. Air temperatures of about 80° F. and higher induce partial to complete masking of the symptoms.

Extensive cytological and histological studies of the different parts of the plant were made, using fresh as well as fixed and stained material. The findings of these studies are as follows:

1. A mycorrhizal fungus was repeatedly found in the small fibrous roots. This fungus has no causal relation to xanthosis, for it is equally common in roots of healthy and of diseased plants. No other organism was found in any of the above-ground parts of the plant.

2. The palisade cells of the chlorotic areas of the leaves are considerably shorter and more compact than those of the healthy plants or of the green areas of the leaves of the diseased plants.

3. A degeneration of the tissue of the pericycle region of the roots was found to be constantly associated with the disease. In this same region cells were often found with partly or completely degenerated nuclei and containing two types of black-staining bodies, designated in this paper as x and y bodies. These cell inclusions display no internal structure, and are considered to be degeneration products. Neither the degenerating tissue nor the cell inclusions were ever found in roots of healthy plants.

Specific and varietal resistance to xanthosis are briefly discussed. The beach strawberry, *Fragaria chiloensis* Duch., is immune, as far

as can be judged by external symptoms, while the wood strawberry, *F. californica* C. & S., is susceptible. The cultivated varieties show varying degrees of resistance, the Marshall and Marshall-like varieties being highly susceptible.

Possible control measures are suggested and discussed.

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# A COMPARATIVE STUDY OF THE GLUTELINS OF THE CEREAL GRAINS<sup>1</sup>

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## INTRODUCTION

When in 1908 the American Society of Biological Chemists (2)<sup>2</sup> drew up the classification of proteins which has come to be known as the American classification, there was included for the first time a group of proteins designated "glutelin." This group is characterized as consisting of "simple proteins insoluble in all neutral solvents but readily soluble in very dilute acids and alkalies." Recognition of the glutelins was brought about chiefly through the work of Osborne and his collaborators, who found that in the case of many of the cereal grains—wheat, corn, oats, rye, and barley—there remained a fraction of nitrogen after extraction of the grain with water, salt solution, and alcohol. It was assumed that this residual nitrogen, which could be extracted by means of alkaline and acid solutions, represented protein. That this is true was demonstrated in the case of wheat, from which a definite alkali-soluble protein, glutenin, may be readily extracted and prepared in a state of high purity. Chittenden and Osborne (7, 1891) and later Osborne and Clapp (35) isolated and studied the glutelin of corn. In 1908 Rosenheim and Kajiura (39) isolated from rice an alkali-soluble protein which they called "oryzenin."

But despite the fact that this class of proteins was included in the American classification, there has remained considerable doubt whether they are at all widely distributed. As lately as 1924 Osborne (34) states that "although it is possible that proteins of the character of glutelins are widely distributed among the different seeds there is no conclusive evidence that this is in fact so." He goes on to say that the only well-defined glutelins are glutenin from wheat, oryzenin from rice, and maize glutelin from maize.

In view of these facts it was deemed desirable to attempt the isolation of proteins of the glutelin type from a number of the cereal grains, and, if success in this were attained, to make a comparative study of the products obtained.

Furthermore, since the glutelins already isolated have been prepared and analyzed by different workers, it seemed highly desirable that as large a number as possible be prepared and analyzed by the same worker, in order that strictly comparable data might be obtained. The work reported in this paper was undertaken with these considerations in mind.

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<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 1118.

## HISTORICAL

## GLUTENIN FROM WHEAT

Glutenin has been known longer and studied more extensively than any other glutelin. The reasons for this are not difficult to find. In the first place, it is probably the most distinctive of the five proteins of wheat flour. It forms, with gliadin, the combination known as gluten, and is generally considered to be the binder which holds the gluten proteins together. Its characteristic property of forming gluten when combined physically with gliadin is the factor responsible for the ability of wheat flour to form a dough from which yeast-leavened bread may be produced.

In the second place, it was long ago suggested that the gliadin-glutenin ratio is largely responsible for the quality of the gluten, and consequently is of the highest importance as a measure of the strength of flour. This naturally led to much investigation of these two proteins, and fairly accurate methods were developed for the quantitative estimation of them. Fleurent (11) fixed the optimum at 75 parts gliadin to 25 parts glutenin. Snyder (42) found the ideal ratio to be 65:35, but he also recognized that the quality factor is probably of more importance than the quantity factor. Blish (4), however, showed that the ratio is much more nearly constant for different flours than had formerly been supposed. Grewe and Bailey (18) examined 17 different flour samples collected from various parts of the United States and Canada. From the data obtained there was no evidence of any substantial variation in the proportion of the gluten proteins present in strong and weak flours. Sharp and Gortner (41) and Blish and Sandstedt (6) found that only small variations occur in the ratio of glutenin to total protein of the flour.

The early history of the investigations of the proteins of flour has been admirably detailed by Osborne (33) and will be passed over lightly. Suffice it to say that the first systematic study of the wheat proteins was made by Ritthausen. The only quantitative chemical method available for comparison of compounds such as proteins was at that time the ultimate analysis. This is of little practical value, and Ritthausen's investigations shed little light upon the subject. Osborne was the next to undertake an extended investigation of the vegetable proteins (33). He used the Fischer method of analysis, which enables one to go a considerable distance in making comparisons of various proteins. After the development of the Van Slyke (43) method for determining the distribution of nitrogen of the acid hydrolysate into various fractions, it became possible to differentiate still more clearly the two proteins gliadin and glutenin (4). Analyses by these methods (33, 4) seem to furnish sufficient evidence to justify the conclusion that glutenin is a chemical entity distinct from gliadin, and not a derivative of the latter through the process of denaturation.

Yet the individuality of glutenin itself has been called into question by Halton (19). Through fractional precipitation of glutenin by the addition of acid to its alkaline solution, he prepared at least two proteins. The separation was effected by adding normal hydrochloric acid to the alkaline solution of glutenin until protein separated out in a flocculent condition. This protein was separated. Further addition of acid to the clear filtrate resulted in a second precipitation of protein. The two fractions so obtained were found to be unlike when their racemization curves were studied. Blish (5),

in an attempt to repeat this work, met with no success. He found, however, that glutenin, if partially racemized, precipitated at a different hydrogen-ion concentration than did the original glutenin. Since Halton had made no record of the hydrogen-ion concentration at which his precipitation occurred, it was not possible to duplicate his experimental conditions. Blish suggested that a portion of Halton's glutenin may have been racemized, which would account for the two fractions he obtained, and he concludes that glutenin represents only one protein.

The subject has been reopened, however, by Csonka and Jones (9), who report the isolation of two glutenin fractions from glutenin. The separation was effected by fractional precipitation from a sodium hydroxide solution by the addition of ammonium sulphate. The differences between the two fractions as shown by analyses were so pronounced that there seems to be little doubt that they represent markedly different substances. The  $\beta$ -glutenin, however, was found to constitute but a relatively small fraction of the total glutenin and, consequently, the value of previous analyses will not be materially lessened for the purpose of comparison unless it be found that the relative amount of the two fractions varies in different flours.

A problem of great importance is that of ascertaining whether or not the glutenin of different sorts of wheat flour varies. This has been attacked from two angles, the chemical and the physical, the latter doubtless being the more precise.

Blish (4) prepared samples of glutenin from a high-grade patent flour and from a biscuit flour, and submitted them to the Van Slyke analysis. The results, which are given in Table 13, led him to the conclusion that the two could not be differentiated chemically. Cross and Swain (8) carried out a similar investigation, using four widely different types of flour. Their data are also shown in Table 13. An inspection of their results leads to the conclusion that there are marked differences in the chemical constitution of the various preparations. However, they interpreted the data as showing that no distinction can be drawn. It may be stated here that in the opinion of the writer their data are of doubtful value in settling this question.

The evidence thus briefly discussed points strongly to the conclusion that glutenins from different types of flour are identical.

Evidence of a different sort is presented by Sharp and Gortner (41) as a result of their investigations of viscosity of flour-in-water suspensions. They found that various flours exhibited different changes in viscosity when treated with acids and alkali. It was shown that these differences must be due to the glutenin, since in leaching the flour with distilled water in order to remove the electrolytes, most of the gliadin was also removed, without, however, causing any substantial modification of the imbibitional capacity of the remaining materials. Without going further into the work of Sharp and Gortner, it may be stated that their conclusion was that "there is an inherent difference in the physicochemical properties of the gluten from strong and weak flours and that these differences are due to the colloidal state of the gluten proteins." They state further that "it has been shown that the differences in colloidal properties apparently reside in the protein, glutenin."

From the papers cited in the foregoing discussion it seems evident that glutenins from various types of flour are indistinguishable by

present methods of chemical analysis, and by means of the measurements of specific rotation, refractive index, and absorption spectra. It seems to be equally evident that the glutenins differ decidedly in respect to colloidal behavior. These two lines of evidence need not be considered opposing. Even though the chemical composition of the proteins were so similar that differences could not be detected by the finest possible analytical technic, very wide variations in colloidal properties might occur.

#### GLUTELIN FROM RYE

Einhof (10) in 1805 made the first study of the rye proteins and considered that there were two, namely, an albumin which coagulated on boiling, and another, soluble in alcohol, which he called "gluten." Von Bibra (3) was the first to recognize an alkali-soluble protein in rye. He called this "casein," but did not analyze it.

Ritthausen (37) described three proteins which he found in rye, namely, albumin, soluble in water; an alcohol-soluble protein, which he called "mucedin"; and an alkali-soluble protein, "gluten-casein." The last named was obtained by extracting the rye meal directly with very dilute potash water, precipitating with acetic acid, and washing with water, alcohol, and ether. He made two preparations and gave as an average for the analyses the following figures: Carbon, 52.14 per cent; hydrogen, 6.93 per cent; nitrogen, 16.38 per cent; sulphur, 1.06 per cent; and oxygen, 23.49 per cent.

No further work seems to have been done on the alkali-soluble protein of rye until Osborne (30, 31, 32) investigated it. He gave the following distribution of the four proteins in the rye kernel:

	Per cent
Leucosin, soluble in water.....	0.43
Gliadin, soluble in alcohol.....	4.00
Edestin and proteose, soluble in salt solution.....	1.76
Insoluble in salt solution.....	2.44
	8.63

The value for the alkali-soluble protein was obtained by difference. He states that—

since rye flour yielded no gluten on washing with water, the proteid remaining in the meal after extracting with salt solution and dilute alcohol, could be obtained only by extracting the residual meal directly with dilute potash water. All attempts, however, to thus prepare this substance resulted only in the production of small preparations of very variable composition. The gum present in the seed dissolved freely in the alkaline solution and made it impossible by any means yet discovered to thoroughly purify the preparations.

He concluded that since rye flour yielded no gluten, this substance is "partly or wholly, other than glutenin." Osborne here is probably using "glutenin" in the sense of a protein having physical properties similar to wheat glutenin, and not in a classification sense.

A review of the literature since the time of Osborne's publication has failed to reveal any further work upon the glutelin of rye.

#### GLUTELIN FROM OATS

There is probably less definite knowledge regarding the proteins of oats than of any other of the cereal grains which have been investigated in this respect.

Norton (28) seems to have been the first to leave any record of work upon the proteins of the oat kernel. He recognized three proteins, viz:

1. Albumin, 0.5 to 2.17 per cent, which was taken up from the epidermis (after starch had been mechanically removed by elutriation with slightly ammoniacal water) by boiling with acetic acid. It was precipitated by neutralizing the solution.

2. Casein or "avenine," as it was named by Johnston, 15.76 to 17.72 per cent, dissolved in slightly ammoniacal water and precipitated by acetic acid.

3. Gluten, 1.33 to 2.46 per cent, which was extracted by alcohol.

Von Bibra (3) found that no gluten could be obtained from oat flour by kneading with water. He recognized four proteins or nitrogenous substances. These were albumin, 1.24 to 1.52 per cent, precipitated by boiling the cold-water extract of the ground oats; casein, 0.15 to 0.17 per cent, the material separating from the hot alcohol extract on cooling; plant gelatin, 3 to 3.25 per cent, the substance soluble in both hot and cold alcohol; and a nitrogenous substance insoluble in water and alcohol, 11.38 to 14.85 per cent.

Kreusler (26) obtained oat gliadin, soluble in weak alcohol, and oat legumin, soluble in very dilute alkali.

After Kreusler's work there seems to have been no further investigation of the oat proteins until Osborne (29) undertook a study of them. Osborne concluded that the protein which is extracted from oats by 0.2 per cent KOH solution is in reality an insoluble modification of the globulin. He makes the following statement:

The proteid extracted, after complete exhaustion of the oats with alcohol of 0.9 specific gravity, by 10 per cent salt solution . . . and that dissolved out by dilute potash . . . have so nearly the same composition as the globulin extracted by salt solution directly, that they may be regarded as originally identical.

Osborne found further that when ground oats are exposed to the action of water only, a large amount of the proteins become insoluble in dilute potash solution, the amount thus rendered insoluble increasing with the duration of the contact with water. One hour's treatment rendered one-half, and 24 hours rendered two-thirds, insoluble in 0.2 per cent KOH solution. The composition of the part soluble in KOH after action of water and removal of the alcohol-soluble protein, was found to be indistinguishable from the globulin soluble in salt solution.

As a result of this investigation, Osborne stated that the proteins present in the oat kernel are: A proteose and an acid albumin, dissolving out in water; a globulin extracted by cold 10 per cent salt solution; and a globulin extracted by means of 10 per cent NaCl solution at 65° C. which separates out from the saline solution upon cooling.

It must, however, be borne in mind that the only means for comparison of proteins available at this time was the elementary or ultimate analysis. It has since been shown that proteins of the most widely varying properties may give elementary analyses which check very closely.

A search of the literature has revealed no further attempt to elucidate this problem until Lüers and Siegert (27) undertook to repeat Osborne's work. They prepared the alcohol-soluble protein, two forms of the globulin, one extracted by cold 10 per cent NaCl solution and one extracted by 10 per cent NaCl solution at 65° C., and the alkali-soluble protein. The last named was prepared by extracting the meal with 0.2 per cent cold KOH solution after it had been previously exhaustively extracted with dilute alcohol. The prepa-

rations were submitted to the Van Slyke nitrogen-distribution analysis. Differences undiscovered by the elementary analyses of Osborne were thus revealed. The only significant differences between the avenalin and the alkali-soluble protein were found in the ammonia and the humin fractions. Curiously enough, the sum of these two fractions was the same for both proteins. Leaving the ammonia and humin fractions out of consideration, there was such close agreement between these two analyses that one is justified in concluding that they represent analyses of the same protein in different degrees of purity. Is it to be decided then, on this evidence, that there is no glutelin in the oat kernel? Osborne was inclined to believe that the avenalin is a changed protein, resulting in some way from the presence of water. It is difficult, however, to believe that such profound differences as were shown by two of the preparations would be produced through this agency.

#### GLUTELIN FROM CORN

Reference to the work on corn is greatly facilitated by the admirable bibliography compiled by Keith (24).

Prior to the investigation by Chittenden and Osborne (7, 1891) practically no work had been done upon the proteins of corn. Ritthausen (38) mentions "maize-fibrin," a substance soluble in alcohol. Weyl (44) stated that the powdered seeds of corn yielded a globulinlike substance soluble in 10 per cent NaCl solution, which after purification by repeated precipitation in water and re-solution in 10 per cent NaCl, coagulates at 75° C. No mention, however, seems to have been made of the alkali-soluble protein.

Chittenden and Osborne (7, 1892) reported that "corn meal, after thorough extraction with salt solution and warm dilute alcohol, yields little proteid matter to dilute solutions of potassium hydroxide (0.2 per cent). The proteins which they reported at that time were three globulins, one or more albumins, and an alcohol-soluble protein.

Osborne and Clapp (35), however, isolated a considerable quantity of the alkali-soluble protein. Regarding it they say:

Of its individuality as a protein substance we, of course, know nothing, for it has heretofore been impossible to obtain any considerable quantity of it in a state of even approximate purity as regards admixture with nonprotein substances.

Since 1907 considerable work has been done on corn proteins, but no record of the isolation and analysis of the corn glutelin has been found.

#### GLUTELIN FROM BARLEY

Prior to Osborne's work, very little had been done in regard to the proteins of barley. Von Bibra (3) names albumin, plant gelatin, and casein as constituents of barley, but gives no particulars concerning them. Presumably the alkali-soluble protein is indicated by the term "casein."

Kreusler made an investigation of the barley proteins, the results of which are given by Ritthausen (38). The "gluten-casein," which evidently corresponds to von Bibra's casein, was prepared by cooling the hot alcoholic extract, whereupon a precipitate settled out. This, when purified by boiling with dilute alcohol and fractionally precipitated from solution in acetic acid, had the following composition: Carbon, 53.84 per cent; hydrogen, 7.16 per cent; and nitrogen, 16.63 per cent.

Osborne (30, 31, 32) found that after extraction of the barley flour with 0.5 per cent salt solution, and then repeatedly with hot 75 per cent alcohol, there still remained 41.7 per cent of original nitrogen in the material. He concluded that since the proteins prepared from barley flour are all so similar to those obtained from wheat flour, this seed also contains a considerable quantity of protein soluble only in dilute alkaline solutions. He stated, however, that—

all attempts . . . to thus prepare it in quantity sufficient to yield preparations of even approximate purity resulted in complete failure.

He adds that—

the previous extraction of the flour to remove the proteids already described seemed to render to a great extent the remaining proteid insoluble in potash water and only insignificant precipitates resulted on neutralizing the extracts. The barley flour also contained a large quantity of gum which rendered the filtration of the alkaline extract very difficult, as this gum dissolved freely in potash water.

It looks as though this had been accepted as the final word upon the subject, for since that time there has been discovered no attempt to isolate the glutelin from this cereal.

#### EXPERIMENTAL

The problem presented was the preparation and analysis of the known glutelins of the cereals wheat, *Triticum vulgare*; rice, *Oryza sativa*; corn, *Zea mays*; and oats, *Avena sativa*; and of the unknown glutelins of the cereals einkorn, *Triticum monococcum*; emmer, *T. dicoccum*; durum wheat, *T. durum*; teosinte, *Euchlaena mexicana*; rye, *Secale cereale*; and barley, *Hordeum vulgare*.

#### NOMENCLATURE

It may be considered that there is no nomenclature for this class of proteins. "Glutenin" has been established by long usage for the glutelin of wheat. "Oryzenin" is a logical name for the glutelin of rice. There remains, however, the necessity for inventing names for the glutelins of these other grains, unless we are to be content to designate them oat-glutelin, rye-glutelin, etc. In selecting suitable names, it is highly desirable to have some distinctive suffix and one that is related in some manner to the generic name of the grain. Accordingly, it is suggested that the glutelins which have been prepared be named as follows:

Source of glutelin	Name suggested
<i>Triticum vulgare</i> .	Glutenin (name in use).
<i>Triticum spelta</i> .	Spelta-glutenin.
<i>Triticum durum</i> .	Duro-glutenin.
<i>Triticum dicoccum</i> .	Dicocco-glutenin.
<i>Triticum monococcum</i> .	Monococco-glutenin.
<i>Secale cereale</i> .	Secalenin.
<i>Avena sativa</i> .	Avenin. <sup>3</sup>
<i>Hordeum vulgare</i> .	Hordenin.
<i>Zea mays</i> .	Zeanin.
<i>Euchlaena mexicana</i> (teosinte).	Teozeanin.
<i>Oryza sativa</i> .	Oryzenin (name in use).

In the succeeding portions of this paper, these names will be used in referring to the various proteins.

<sup>3</sup> Abderhalden and Hämmäläinen (1) used the term "avenin" to designate the mixture of proteins prepared by extracting oats with NaOH solution without previous removal of albumin, globulin, and prolamine. It should be reserved for the glutelin alone.

## PREPARATION AND ANALYSIS OF THE PROTEINS

## METHOD

In the isolation of glutelins as described by Osborne, the solutions of the protein in 0.2 per cent alkali were filtered clear. Filtering the alkaline solutions was found impracticable in the present experiments, however, on account of the large volumes of liquid necessary in the extraction of some of the proteins. Consequently the super-centrifuge was called into use. Unfortunately, however, for the uniformity of the procedure, the machine went out of order toward the end of the work; so that three of the proteins, monococco-glutenin, hordenin, and oryzenin, had to be treated in a manner different from the others. A detailed description of the methods of preparation is given, however, for the individual proteins.

All extractions were carried out at room temperature, the materials being well protected with toluene.

All nitrogen determinations were made by the Kjeldahl-Gunning method.

Moisture was determined by heating at 102° C. for two hours in a Freas oven at atmospheric pressure. This method gives reproducible results, and checks fairly closely the vacuum-oven procedure. The samples, after drying, were ashed in platinum at approximately 555° C.

The nitrogen distribution was carried out in general as described by Van Slyke (43). In all cases the proteins were hydrolyzed with 60 c. c. of 20 per cent hydrochloric acid for 24 hours, and filtered immediately at the end of this time. The analyses were performed on duplicate solutions, A and B, which are so indicated in the tables.

Modifications introduced were as follows: The acid-insoluble humin was filtered off after hydrolysis and its nitrogen content determined. The acid-soluble humin was determined after the ammonia nitrogen determination. The nitrogen in the precipitate of barium phosphotungstate from the solution of the bases, after very thorough washing, was determined by the Kjeldahl method, and its nitrogen content reported as phosphotungstic acid humin nitrogen. In precipitating the bases, the solutions were heated to near the boiling point before adding the hot 15 per cent phosphotungstic acid solution. No immediate precipitation occurred in this case, but upon slowly cooling to room temperature, a very dense crystalline precipitate settled out. Thereafter the solutions were kept for 48 hours in an ice-cooled refrigerator. Arginine was determined by the use of the apparatus described by Holm (23).

In making calculations, no corrections were made for the solubility of the phosphotungstates of the bases.

## SOURCE OF PROTEINS

The materials used in the preparation of these proteins were the residues from the alcoholic extraction of the prolamines by Hoffman and Gortner (21). These residues had been dried in drawers over steam pipes, and then stored in cans. This material was used rather than fresh samples in order to avoid the rather laborious task of removing the alcohol-soluble proteins prior to extraction of the glutelins. Subsequent experience, however, has led to the conclu-

sion that time might have been saved by beginning with fresh grains, since these residues had become so hard, due to desiccation, that it required, in some cases, weeks in order to get them soaked properly.

#### GLUTENIN FROM WHEAT

Glutenin was prepared by a modification of Osborne's (33) method. There was placed 690 gm. of the crude glutenin (residue from Hoffman's extraction of gliadin) in a 5-gallon stoneware jar with 18 to 20 liters of 0.2 per cent sodium hydroxide solution, and this was stirred frequently for one day. After allowing the mixture to stand undisturbed for 24 hours, 12 liters of the clear solution was removed by siphoning, and this was replaced by 12 liters of fresh 0.2 per cent NaOH solution. This procedure was repeated three times, until all the solid material was dispersed.

The solutions as removed were neutralized with 0.2 per cent HCl until the precipitate settled, leaving an absolutely water-clear supernatant liquid. This was considered to be the point of maximum flocculation of the protein. The precipitates thus obtained were collected and redispersed in 5 to 6 liters of 0.2 per cent NaOH solution. As soon as possible after the addition of the alkali, the solution was put through a Sharples supercentrifuge, 40,000 revolutions per minute, at the medium rate of feed. It was then put through again at the slowest rate of feed, which gives the maximum separation attainable with the machine. These solutions did not, even with this treatment, come out absolutely clear, but always showed a slight opalescence. This, of course, would be expected, since the volume of NaOH solution used in the second dispersion was kept as low as possible in order to save time in centrifuging. The solutions were finally diluted by the addition of an equal volume of distilled water and the protein precipitated by the addition of 0.2 per cent HCl.

After allowing the precipitate to settle, the supernatant liquid was siphoned off and 3 to 4 liters of recovered ethyl alcohol (85 per cent) was added. This was stirred frequently for one day and then decanted off. There followed six successive similar washings with 95 per cent ethyl alcohol. This, it was thought, removed all traces of gliadin. Analysis of the alcohol after the fourth washing showed 0.0017 per cent nitrogen. The protein was washed twice more after this and finally four successive times with 1 to 2 liters of ether. It was then filtered on a heavy cloth, dried for 36 hours at approximately 60° C., and finally ground in a ball mill for 5 hours. The white powder so obtained was subjected to a further drying of 48 hours at 50°-60° in a Freas oven. It was analyzed with the following results:

	Per cent
Moisture.....	0.99
Ash.....	1.01
Nitrogen.....	16.31
Nitrogen corrected for ash and moisture.....	16.64

Table 1 shows the nitrogen distribution of the glutenin thus prepared.

TABLE 1.—*The nitrogen distribution of glutenin as determined by the Van Slyke method expressed as percentages of total nitrogen*

Nitrogen	A	B	Mean
Ammonia N.....	14.63	14.93	14.78
Total human N.....	1.85	1.55	1.70
Acid insoluble.....	.64	.41	.52
Acid soluble.....	.46	.34	.40
Phosphotungstic.....	.75	.80	.78
Total basic N.....	17.96	19.65	18.80
Arginine.....	10.80	11.00	10.90
Cystine.....	.44	.37	.40
Lysine.....	5.34	6.32	5.83
Histidine.....	1.38	1.96	1.67
Total filtrate N.....	63.60	63.47	63.54
Amino.....	58.38	57.92	58.15
Nonamino.....	5.22	5.55	5.39
Total.....	98.04	99.60	98.82

By reference to the first two entries in Table 13 it will be noted that these figures in Table 1 agree fairly well with those obtained by Blish, except for the histidine, lysine, and nonamino nitrogen of the filtrate. Either the values for the amino nitrogen determinations are too high in the writer's data or they are too low in those of Blish. In respect to the writer's data, it is considered that A is in error in the basic fraction. The values shown under B are more nearly correct than the mean value. As will be shown subsequently, there is no justification for accepting differences in check determinations as great as those given here. Since all the other fractions check very well, it is assumed that loss of material occurred during the concentration of the solution of the bases.

#### SPELTA-GLUTENIN FROM SPELT

This protein was prepared by extracting 680 gm. of the spelt residue five successive times with 12-liter portions of 0.2 per cent NaOH solution. The duration of the extraction periods was 1, 5, 5, 6, and 11 days, respectively. The solutions as siphoned off were quite clear and yielded only very small precipitates. The five precipitates thus obtained were collected and redispersed in about 7 liters of 0.2 per cent NaOH solution, and within 30 minutes the liquid was put through the supercentrifuge at the slow rate. The liquid came through quite clear, but was a deep orange color. In the bowl of the centrifuge there was left a considerable quantity of almost white material of a gummy nature. Starch thrown out of suspension at 40,000 revolutions per minute forms a dry hard cake on the walls of the centrifuge bowl, and is nearly all found at the lower end of it, but this material was quite soft, even slimy, and extended most of the length of the bowl. It was removed and triturated with alcohol, washed several times with 95 per cent alcohol, and finally with ether. After drying, it was ground in a mortar. There was thus obtained a white powder, which showed upon analysis a nitrogen content of 0.87 per cent.

The clear solution, upon addition of HCl, yielded a light brown precipitate of the protein. This was treated in the manner described under glutenin. Color was extracted by all treatments with alcohol,

but no color appeared in the ether. After drying at 60° C. and grinding in the ball mill, 16 gm. of fawn-colored powder was obtained, which was analyzed, with the following results:

	Per cent
Moisture.....	6.55
Ash.....	1.03
Nitrogen.....	11.29
Nitrogen corrected for ash and moisture.....	12.21

Table 2 shows the nitrogen distribution of spelta-glutenin.

TABLE 2.—*The nitrogen distribution of spelta-glutenin as determined by the Van Slyke method, expressed as percentages of total nitrogen*

Nitrogen	A	B	Mean
Ammonia N.....	7.77	8.34	8.06
Total humin N.....	5.12	4.93	5.02
Acid insoluble.....	2.76	2.52	2.64
Acid soluble.....	1.26	1.19	1.22
Phosphotungstic.....	1.10	1.22	1.16
Total basic N.....	24.30	24.57	24.44
Arginine.....	13.11	13.75	13.43
Cystine.....	.34	.40	.41
Histidine.....	2.76	2.83	2.80
Lysine.....	8.09	7.50	7.80
Total filtrate N.....	62.45	61.68	62.07
Amino.....	56.40	56.20	56.35
Nonamino.....	5.96	5.48	5.72
Total.....	99.64	99.52	99.59

#### DURO-GLUTENIN FROM DURUM WHEAT

The protein was prepared by extracting 620 gm. of the crude glutenin with 18 to 20 liters of 0.2 per cent NaOH solution, and afterwards the seven successive 12-liter portions in the manner heretofore described. The duration of the extraction periods was 3, 2, 4, 5, 7, 6, 4, and 6 days, respectively. The first two batches of solution were quite clear and were not centrifuged, but all the later ones were put through the supercentrifuge as they were siphoned from the extraction jar. The first five precipitates obtained were combined and worked up in the manner described under glutenin, and the last three were collected and worked up separately.

As in the case of the spelta-glutenin, there was obtained during the supercentrifuging a white gummy residue which was retained. The residues from all the different centrifugings were combined and extracted for 10 days with 8 liters of 0.2 per cent NaOH solution. This was then decanted off and the residue washed with alcohol and ether and dried. The nitrogen content of the residue was found to be 0.62 per cent.

The two batches of protein varied in nitrogen content, No. 1 having 15.5 per cent and No. 2, 14.2 per cent. The material was almost white, and upon analysis gave the following values:

	Per cent
Moisture.....	1.78
Ash.....	.28
Nitrogen.....	15.15
Nitrogen corrected for ash and moisture.....	15.46

Table 3 shows the nitrogen distribution of duro-glutenin, preparation 1.

TABLE 3.—*The nitrogen distribution of duro-glutenin as determined by the Van Slyke method, expressed as percentages of total nitrogen*

Nitrogen	A	B	Mean
Ammonia N.....	13.11	13.40	13.25
Total humin N.....	2.42	2.56	2.49
Acid insoluble.....	.87	.81	.84
Acid soluble.....	.84	.74	.79
Phosphotungstic.....	.71	1.01	.86
Total basic N.....	21.13	21.28	21.20
Arginine.....	10.44	11.56	11.00
Cystine.....	.95	.81	.88
Histidine.....	6.33	5.36	5.84
Lysine.....	3.41	3.55	3.48
Total filtrate N.....	63.26	63.69	63.48
Amino.....	54.57	54.37	54.47
Nonamino.....	8.69	9.32	9.01
Total.....	99.92	100.93	100.42

#### DICOCO-GLUTENIN FROM EMMER

In the extraction of dicocco-glutenin 2,640 gm. of the Hoffman residue was used in two separate batches. The solutions as siphoned off were fairly clear and were not supercentrifuged. The first extraction was of 8 days duration, the second 13 days, and the third 9 days, for each batch. At the end of this time the amount of precipitate obtained was negligibly small and the extractions were discontinued. The crude protein obtained from the first extraction of 8 days was combined and worked up as preparation 1, while the combined precipitates of the following two extractions were worked up later as preparation 2. On redissolving, 5 liters of NaOH solution was used. The solution was immediately put through the supercentrifuge twice. In this case no significant amount of gummy material was obtained. There was a small amount of starch thrown out in the lower part of the bowl. The solution as it finally came from the supercentrifuge was practically water-clear and was considered one of the best examples of separation so far obtained. The two preparations analyzed for nitrogen 14.23 and 13.45 per cent, respectively. There was no difference in the appearance of these two preparations, and so far as it could be controlled, the technic of preparation was the same in each case. Preparation 1 was used in the following analyses:

	Per cent
Moisture.....	1.52
Ash.....	.83
Nitrogen.....	14.23
Nitrogen corrected for ash and moisture.....	14.58

Table 4 shows the nitrogen distribution of dicocco-glutenin, preparation 1.

TABLE 4.—*The nitrogen distribution of dicocco-glutenin as determined by the Van Slyke method, expressed as percentages of total nitrogen*

Nitrogen	A	B	Mean
Ammonia N.....	11.04	11.07	11.06
Total humin N.....	4.41	4.84	4.63
Acid insoluble.....	1.47	1.45	1.46
Acid soluble.....	1.87	2.44	2.16
Phosphotungstic.....	1.07	.95	1.01
Total basic N.....	24.96	24.24	24.60
Arginine.....	13.03	13.03	13.03
Cystine.....	.79	.71	.75
Histidine.....	5.33	4.26	4.80
Lysine.....	5.81	6.24	6.02
Total filtrate N.....	59.07	59.59	59.34
Amino.....	54.68	54.13	54.41
Nonamino.....	4.39	5.46	4.93
Total.....	99.48	99.74	99.63

## MONOCOCCO-GLUTENIN FROM EINKORN

Einkorn residue to the amount of 1,830 gm. was used in this preparation in one batch. Periods of extraction were 6, 3, 10, and 10 days, respectively. The precipitates as collected were kept under alcohol of approximately 50 per cent strength, which was changed at intervals of 3 or 4 days. This extracted a considerable amount of reddish coloring material. In no case was the precipitate obtained from the 12 liters of solution very great in quantity. After redissolving the collected precipitates, it was found that the supercentrifuge was out of order, and it was decided to try filtering the solution. Previous experience had shown that filtration through ordinary filter paper removed very little of the suspended material. A heavy duck filter cloth was tried, but it was found that in a very short time the rate of filtration became so slow that this method was impractical. A layer of filter paper pulp, 1 to 1½ inches in thickness on a Büchner funnel was then tried, using a suction pump. This method was satisfactory for a small amount of solution, say the first 2 liters, but the mat rapidly became clogged and even in vacuum of 30 mm. Hg the rate of flow was impossibly slow. To overcome this difficulty, recourse was had to the addition of alcohol to the alkaline solution of the protein. An equal volume of 95 per cent methyl alcohol was added, and this solution filtered with reasonable rapidity. It was filtered twice, and the resultant solution was practically water-clear and of light brown color.

The subsequent treatment of this protein was somewhat different from those heretofore described, because time had become a matter of importance. A full description of it will be given, since two other proteins were prepared in the same manner, namely, hordenin and oryzenin.

Dilute acetic acid was added to the clear filtrate, until it was judged that optimum precipitation had occurred. The precipitate was allowed to settle for approximately an hour, the supernatant liquid decanted, and the remainder of the material poured out upon a heavy duck filter cloth and allowed to drain until most of the liquid

had passed off. The cloth was then gathered up in the hands and as much of the remaining liquid as possible was removed by twisting the cloth. The protein was then removed from the cloth with a steel spatula and triturated in a large porcelain mortar with absolute ethyl alcohol. This was allowed to stand with frequent stirring for 24 hours and the process repeated. After three such treatments ether was substituted for alcohol, and it was found that two treatments of 24 hours' duration with ether were sufficient to desiccate the protein to such an extent that it would dry to a powder in the course of 3 to 6 hours at room temperature. When most of the ether had evaporated, the protein was lightly rubbed in a mortar and placed in a Freas oven at 50–60° C., and allowed to dry for 24 hours.

This method of purifying the proteins seems to involve considerable loss. While there was not a great deal of crude protein originally extracted, the amount finally obtained, namely, 5 gm., was disappointingly small. It is probable that precipitation from the alcoholic NaOH solution is not as complete as from the aqueous NaOH solution. This, however, is conjecture, and is a matter for further investigation.

The product obtained was an exceedingly fine powder of light brown color, which upon analysis gave the following results:

	Per cent
Moisture.....	1.43
Ash.....	1.07
Nitrogen.....	13.23
Nitrogen corrected for ash and moisture.....	13.57

Table 5 shows the nitrogen distribution of monococco-glutenin.

TABLE 5.—*The nitrogen distribution of monococco-glutenin as determined by the Van Slyke method, expressed as percentages of total nitrogen*

Nitrogen	A	B	Mean
Ammonia N.....	10.80	10.76	10.78
Total humin N.....	6.03	6.05	6.04
Acid insoluble.....	2.69	2.68	2.69
Acid soluble.....	2.30	2.24	2.27
Phosphotungstic.....	1.04	1.13	1.08
Total basic N.....	20.09	20.22	20.16
Arginine.....	11.74	11.97	11.86
Cystine.....	1.09	1.00	1.04
Histidine.....	2.47	2.40	2.44
Lysine.....	4.79	4.85	4.82
Total filtrate N.....	63.28	63.25	63.26
Amino.....	56.74	56.59	56.66
Nonamino.....	6.54	6.66	6.60
Total.....	100.20	100.28	100.24

#### SECALENIN FROM RYE

In the preparation of this protein a slight departure was made from the ordinary routine. It was thought desirable to ascertain, if possible, whether or not there might be different kinds of protein extracted during the course of the long-continued treatments with alkaline solutions, which seem to be necessary in order to extract the protein material from the residues from the alcoholic extraction.

Accordingly it was decided to collect five separate fractions. The first four fractions were taken off after successive periods of extraction of 24 hours  $\pm$  1 hour, while the fifth fraction contained all the protein collected in subsequent extractions. Thus the preparations would result from exposure to the NaOH solution for periods of 1, 2, 3, 4, and 5 to 7 days. In all cases very small quantities of protein were obtained from the solutions as siphoned off from the extraction vessels. The volume of NaOH solution used was so large that no attempt was made to centrifuge at this time. Instead, it was thought advisable to collect the crude precipitates and redissolve them in as small volumes as possible and then centrifuge them very thoroughly. This was accordingly attempted. It was found, however, that in trying to put the collected precipitates back into solution a residue always remained, that is to say, clear solutions such as the original extracts could not be obtained. The mixture was, however, passed through the centrifuge, and it was found that the insoluble portion was more or less completely thrown out of suspension on the walls of the centrifuge bowl. This substance, which was encountered here for the first time in significant quantity, did not look like starch, since it was soft and slimy rather than hard and caked as starch would be after centrifuging. Not much attention was paid to this material at first, but when the same phenomenon was noted with successive fractions, it was thought advisable to keep the material in case it might prove to be protein. A sample of this substance was dried with alcohol and ether and analyzed. It contained 0.21 per cent nitrogen. It was further observed that this material might be dispersed in concentrated alkali solution to form a light brown highly viscous solution or dispersion. This on neutralization gave a flocculent white precipitate which settled very slowly. On the addition of 85 per cent alcohol the material evidently flocculated further, and settled more rapidly. It was washed three successive times with alcohol and finally with ether. After some washings with ether the precipitate had conglomerated and become gellike, so that it could be lifted out by hand. It could be stretched and kneaded like gluten, which, in fact, it resembled considerably. After the ether had evaporated, the exterior of the mass became sticky, and finally dried down to a dark brown horny mass, which ground up to a pure white powder. It was concluded that this material is probably the "gummy substance" referred to by Osborne, and may be responsible for the difficulty hitherto encountered in the preparation of the glutelin of rye.

Unfortunately the significance of the gummy material described above did not become apparent until after most of the fractions of the rye protein had been prepared. Consequently there is no check upon the length of time which elapsed between the redissolving of the crude protein and the centrifuging of it, and it is thought that variations in this period might have been responsible for the varying degrees of purity of the preparations obtained.

The five preparations gave upon analysis a nitrogen content of 12.00, 13.30, 15.15, 13.02, and 11.05 per cent, respectively. In an attempt to redissolve preparation 1 in order to undertake further purification of it, the whole sample was lost. The amounts of protein were small, ranging from 10 to 15 gm. each. The preparations

varied in color from light cream for Nos. 3 and 5, light brown for No. 2, to dark brown for No. 4. Analyses of the four fractions gave the following results:

Preparation	Ash	Moisture	Nitrogen	Nitrogen corrected for ash and moisture
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
2	0.56	2.19	13.30	13.67
3	.50	2.46	15.15	15.61
4	.38	1.75	13.02	13.30
5	19.16	2.60	11.05	14.12

The preparations 2, 3, 4, and 5 were submitted to the Van Slyke method of analysis, but only the results for 2, 3, and 5 are given in Table 6, since No. 4 was ruined.

TABLE 6.—The nitrogen distribution of secalenin as determined by the Van Slyke method, expressed as percentages of total nitrogen

Nitrogen	Preparation 2			Preparation 3			Preparation 5		
	A	B	Mean	A	B	Mean	A	B	Mean
Ammonia N.....	9.86	9.69	9.78	10.70	10.91	10.80	7.05	7.22	7.14
Total humin N.....	5.44	5.14	5.29	4.46	4.15	4.30	4.40	4.21	4.30
Acid insoluble.....	2.18	2.17	2.17	1.53	1.53	1.53	1.44	1.32	1.38
Acid soluble.....	2.16	1.86	2.01	1.80	1.66	1.73	2.03	2.00	2.01
Phosphotungstic.....	1.10	1.11	1.11	1.13	.96	1.04	.93	.89	.91
Total basic N.....	25.54	26.22	25.88	25.40	25.40	25.40	25.79	25.79	25.79
Arginine.....	13.58	13.73	13.65	13.98	13.74	13.86	13.85	13.75	13.80
Cystine.....	.00	.33	.47	.70	.65	.68	.63	.56	.60
Histidine.....	5.19	5.25	5.22	5.11	5.39	5.25	3.67	3.66	3.66
Lysine.....	6.17	6.91	6.54	5.61	5.62	5.61	7.64	7.82	7.73
Total filtrate N.....	58.16	58.51	58.34	59.50	59.49	59.49	62.29	62.71	62.50
Amino.....	51.66	52.21	51.94	52.70	52.78	52.74	57.94	58.35	58.14
Nonamino.....	6.50	6.30	6.40	6.80	6.71	6.75	4.35	4.36	4.36
Total.....	99.00	99.56	99.29	100.06	99.95	99.99	99.53	99.93	99.73

#### AVENIN FROM OATS

Extraction of 8 kgm. of material was carried on in the manner described under glutenin. All the crude precipitates were collected and worked up together. The whole was dispersed in 30 liters of 0.2 per cent NaOH solution and passed through the super-centrifuge twice. Considerable amounts of starch and some gummy material were thus removed. Unfortunately the gummy material was not kept. The resultant solution was fairly clear. It was diluted about 1:1 with distilled water, and the protein precipitated by means of 0.2 per cent HCl solution. The supernatant liquid was decanted off and the residue washed five successive times with 95 per cent alcohol, using about 6 liters per washing. It was finally extracted with 3 liters of ether, drained on a filter cloth, dried in the air for 24 hours, finally over steam pipes for 40 hours, ground in a ball mill, and again dried over the steam pipes for 14 hours. There was

obtained 250 gm. of a light, cream-colored material, which upon analysis gave the following results:

	Per cent
Moisture.....	2.04
Ash.....	1.41
Nitrogen.....	15.23
Nitrogen corrected for ash and moisture.....	15.77

Table 7 shows the nitrogen distribution of avenin.

TABLE 7.—*The nitrogen distribution of avenin as determined by the Van Slyke method, expressed as percentages of total nitrogen*

Nitrogen	A	B	Mean
Ammonia N.....	11.91	11.82	11.87
Total humin N.....	3.67	3.38	3.53
Acid insoluble.....	1.50	1.19	1.35
Acid soluble.....	1.14	1.32	1.23
Phosphotungstic.....	1.03	.87	.95
Total basic N.....	24.71	24.04	24.37
Arginine.....	15.50	15.47	15.48
Cystine.....	.93	1.28	1.10
Histidine.....	3.24	3.21	3.23
Lysine.....	5.04	4.08	4.56
Total filtrate N.....	58.59	60.99	59.94
Amino.....	56.65	58.79	57.72
Nonamino.....	2.24	2.20	2.22
Total.....	99.18	100.23	99.71

#### ZEANIN FROM MAIZE

Three kgm. of crude zeanin, residue from the extraction of zein from corn gluten was extracted seven successive times with 0.2 per cent NaOH solution. Collected precipitates were redissolved in 0.2 per cent NaOH, supercentrifuged, and washed with alcohol and ether in the usual manner. An analysis of this product, which was a light cream in color, gave the following results:

	Per cent
Moisture.....	1.53
Ash.....	4.70
Nitrogen.....	13.58
Nitrogen corrected for ash and moisture.....	14.49

Table 8 gives the nitrogen distribution of zeanin. These results represent two separate analyses. B and C were run concurrently.

TABLE 8.—*The nitrogen distribution of zeanin as determined by the Van Slyke method, expressed as percentages of total nitrogen*

Nitrogen	A	B	C	Mean
Ammonia N.....	11.66	11.07	11.24	11.32
Total humin N.....	2.76	2.45	2.54	2.58
Acid insoluble.....	1.18	1.02	1.06	1.09
Acid soluble.....	.50	.71	.87	.79
Phosphotungstic.....	.78	.72	.61	.70
Total basic N.....	19.66	18.31	18.57	18.84
Arginine.....	9.40	9.66	9.29	9.45
Cystine.....	.74	.99	1.27	1.00
Histidine.....	4.26	3.27	4.18	3.90
Lysine.....	5.26	4.39	3.83	4.49
Total filtrate N.....	66.36	67.54	67.35	67.08
Amino.....	60.93	61.67	61.85	61.48
Nonamino.....	5.43	5.87	5.50	5.60
Total.....	100.44	99.37	99.70	99.82

## TEOZEANIN FROM TEOSINTE (ANNUAL OR FLORIDA)

Thirty kgm. of teosinte from which the teozein had been removed was extracted in batches of about 2 kgm. each with 0.2 per cent NaOH solution. Four successive extractions of 24 hours duration each were made on each batch. The initial solutions were not centrifuged. The collected precipitates were redissolved in 0.2 per cent NaOH solution and put through the supercentrifuge twice. Considerable amounts of starch were removed, and the resultant solutions were quite clear and very deeply colored. The clear solution was diluted with an equal volume of distilled water and the protein reprecipitated by the addition of 0.2 per cent HCl. It was then dried by treatment with 95 per cent alcohol and ether. Seventy-eight grams of an almost black powder was obtained, which upon analysis gave the following results:

	Per cent
Moisture.....	1.60
Ash.....	2.39
Nitrogen.....	10.54
Nitrogen corrected for ash and moisture.....	11.02

Table 9 gives the nitrogen distribution of teozeanin.

TABLE 9.—*The nitrogen distribution of teozeanin as determined by the Van Slyke method, expressed as percentages of total nitrogen*

Nitrogen	A	B	Mean
Ammonia N.....	10.32	9.99	10.16
Total humin N.....	8.31	8.23	8.27
Acid insoluble.....	5.68	5.66	5.67
Acid soluble.....	1.61	1.50	1.55
Phosphotungstic.....	1.02	1.07	1.05
Total basic N.....	26.63	27.07	26.85
Arginine.....	14.17	14.14	14.15
Cystine.....	1.21	1.16	1.18
Histidine.....	7.89	8.07	7.98
Lysine.....	3.36	3.70	3.53
Total filtrate N.....	55.22	54.73	54.97
Amino.....	49.69	49.62	49.65
Nonamino.....	5.53	5.11	5.32
Total.....	100.48	100.02	100.25

## HORDENIN FROM BARLEY

In the first attempt to extract the glutelin from barley meal, 2 kgm. of the Hoffman residue was treated for 24 hours with 20 liters of 0.2 per cent NaOH solution. After settling, 12 liters of the supernatant liquid was siphoned off and 0.2 per cent HCl solution was added. It was observed that the solution turned milky after the NaOH had been neutralized, but no precipitate settled out, and no amount of adjusting of the acidity of the solution met with any success. It looked as if protein were present, but could not be flocculated, and the attempt at this time was abandoned.

After the other proteins heretofore described had been prepared, another attempt was made to isolate the glutelin from barley. About

2 kgm. of Hoffman's residue was stirred up with 16 liters of 0.2 per cent NaOH solution. Vigorous stirring at frequent intervals was continued for about five hours. After this time, the residue had become extremely viscous. Since the centrifuge was not working, it looked as if it would be impossible to remove the substance which was responsible for the high viscosity of the mixture. In the hope of bringing the liquid into condition to be filtered through paper pulp, a portion was withdrawn and an equal volume of 95 per cent alcohol added to it. A white, fibrous coagulum resulted from the mixing with the alcohol. In the course of an hour this settled, and the clear, slightly colored, supernatant liquid was decanted off and filtered through a heavy layer of filter paper pulp, using very slight suction. A perfectly clear, golden brown solution was obtained. This, upon treatment with dilute acetic acid, yielded a flocculent, light gray precipitate. About 12 liters of the original extract was treated in this manner, the time required being five days. Collected precipitates of the various small batches of filtrate were redissolved, filtered, and reprecipitated. This second filtration gave no clearer solution than the first, and it is doubtful whether any great degree of purification was effected by this operation. The protein was treated in the manner described for monococco-glutenin. Thirteen grams of an almost white powder was obtained. This upon analysis gave the following values:

	Per cent
Moisture.....	1.34
Ash.....	.69
Nitrogen.....	15.30
Nitrogen corrected for ash and moisture.....	15.62

The white material which was precipitated by the addition of alcohol to the original extract appeared to be of a gummy nature. It was slightly soluble in cold water, but was readily dispersed in hot water, giving an opalescent suspension which resembled a suspension of soluble starch. This suspension gave a marked starch reaction with iodine, but this was due to traces of starch which must have been very small, since the addition of a very little ptyalin in the form of saliva caused the disappearance of the starch reaction.

Table 10 gives the nitrogen distribution of hordenin.

TABLE 10.—*The nitrogen distribution of hordenin as determined by the Van Slyke method, expressed as percentages of total nitrogen*

Nitrogen	A	B	Mean
Ammonia N.....	11.48	11.28	11.38
Total humin N.....	3.51	3.02	3.26
Acid insoluble.....	1.42	1.39	1.40
Acid soluble.....	1.39	1.05	1.22
Phosphotungstic.....	.70	.58	.64
Total basic N.....	20.22	20.28	20.25
Arginine.....	10.88	11.27	11.08
Cystine.....	1.88	1.70	1.79
Histidine.....	2.40	1.70	2.05
Lysine.....	5.06	5.61	5.33
Total filtrate N.....	64.50	64.52	64.51
Amino.....	57.69	57.65	57.67
Nonamino.....	6.81	6.87	6.84
Total.....	99.71	99.10	99.40

## ORYZENIN FROM RICE

Two 1,000-gm. batches of polished rice meal which had been extracted with alcohol were treated with 18-liter portions of 0.2 per cent NaOH solution. After 24 hours, 12 liters of the clear supernatant liquid was siphoned off and the protein precipitated by the addition of dilute acetic acid. Three successive extractions of each batch were thus made. The amounts of precipitate obtained were small. The collected precipitates were redissolved and an equal volume of 95 per cent alcohol added. The solution was filtered twice through a mat of filter paper pulp, using slight suction. This gave a perfectly clear, light yellow solution, which upon addition of dilute acetic acid yielded a white flocculent precipitate of the protein. This was dried by means of absolute alcohol and ether. The resultant material required no grinding, but disintegrated to a white fluffy powder. Thirteen grams was obtained. This gave, upon analysis, the following values:

	Per cent
Moisture.....	1.97
Ash.....	.97
Nitrogen.....	15.93
Nitrogen corrected for ash and moisture.....	16.41

Table 11 gives the nitrogen distribution of oryzenin.

TABLE 11.—*The nitrogen distribution of oryzenin as determined by the Van Slyke method, expressed as percentages of total nitrogen*

Nitrogen	A	B	Mean
Ammonia N.....	7.92	8.22	8.07
Total humin N.....	1.89	2.02	1.95
Acid insoluble.....	.53	.59	.56
Acid soluble.....	.47	.69	.58
Phosphotungstic.....	.89	.74	.81
Total basic N.....	27.30	27.89	27.59
Arginine.....	17.45	18.46	17.95
Cystine.....	.70	.53	.61
Histidine.....	4.84	4.35	4.60
Lysine.....	4.31	4.55	4.43
Total filtrate N.....	63.00	62.05	62.52
Amino.....	55.94	55.99	55.96
Nonamino.....	7.06	6.06	6.56
Total.....	100.11	100.18	100.13

## DISCUSSION

Before discussing the results obtained, it might be advantageous to consider the value of the analytical method employed.

The experience gained in the work here recorded has led the writer to believe that the errors most likely to occur in analyses by the Van Slyke method are concerned chiefly with the amino nitrogen determination. Here, very slight errors profoundly affect the values obtained for histidine, lysine, and for the nonamino nitrogen of the filtrate from the bases. The values upon which the highest reliance, in the analytical sense, is placed, are the ammonia nitrogen, humin nitrogen, arginine nitrogen, total basic nitrogen, and total nitrogen

in the filtrate from the bases; in other words, essentially the Hausmann numbers and the arginine value. This does not mean that these are necessarily the true values for the pure protein, but that for the material in hand they can be determined with a high degree of accuracy.

Since the conclusions to be drawn from the analytical data involve certain assumptions regarding the various nitrogen fractions determined, it is necessary here to scrutinize rather carefully the methods used in making these analyses, giving particular attention to what is known regarding the effect of impurities upon the distribution of nitrogen in the various fractions. The humin fraction will be discussed first, because it is the one most profoundly affected by impurities.

Gortner and Holm (16), after a long series of experiments (12-17) upon humin, concluded that the acid-insoluble humin occurs as a result of interaction of tryptophane and some unknown substance of an aldehydic nature; the acid-soluble humin is due to interaction of tyrosine and the aldehyde, while the phosphotungstic acid humin is probably due to adsorption of monoamino acids on the barium phosphotungstate. Starch and other carbohydrates also affect the humin fraction. This may be accounted for as due partly to the formation of furfural, which acts through its aldehyde group, and partly to the formation of the large amount of "humuslike" material which acts as a nonspecific adsorbent. If this were the complete explanation for the formation of humin neither the ammonia nor the basic nitrogen fractions should be very greatly affected by the presence of impurities, which would increase the humin fraction. The net result of an increase of humin nitrogen would be a corresponding decrease of the amino nitrogen of the filtrate from the bases. But experiment does not bear out these conclusions.

TABLE 12.—Effect of the presence of carbohydrates on the distribution of nitrogen

Item	Ammonia N	Humin N	Nitrogen of bases					Nitrogen in filtrate from bases		Total recovery	Observer
			Arginine	Cystine	Histidine	Lysine	Total	Amino	Nonamino		
Lactalbumin.....	P. ct. 8.57	P. ct. 2.32	P. ct. 7.20	P. ct. 1.30	P. ct. 4.57	P. ct. 12.24	P. ct. 25.31	P. ct. 52.00	P. ct. 2.00	P. ct. 100.20	Osborne, Van Slyke, Leavenworth, and Vinograd (36). Do.
7 gm. lactalbumin+7 gm. glucose.	8.37	3.70	8.10	1.05	3.22	12.54	24.91	58.79	3.58	99.35	
3 gm. fibrin.....	10.15	2.83	10.91	.51	4.36	12.05	27.83	55.43	2.51	98.75	Gortner (12). Do.
3 gm. fibrin+9 gm. cellulose.	9.85	7.72	8.56	.71	4.86	11.04	25.17	52.02	3.91	98.67	
Casein.....	10.27	1.28	7.41	.20	5.76	10.70	24.07	55.81	7.13	98.56	Van Slyke (45). Hart and Sures (30) Do.
Do.....	10.37	1.47	7.95	.19	5.95	9.41	23.50	59.32	7.85	102.51	
2.4 gm. casein+12 gm. glucose.	10.09	3.76	7.59	.19	7.31	7.01	22.10	57.85	8.89	102.71	Do.
2.4 gm. casein+12 gm. sucrose.	8.54	9.15	6.77	.19	7.65	6.38	20.99	55.38	9.28	103.38	
2.4 gm. casein+12 gm. starch.	9.20	7.46	6.74	.19	7.30	5.54	19.77	50.89	13.76	101.10	Do.
2.4 gm. casein+6 gm. xylan.	10.40	11.83	5.56	.19	6.52	8.55	19.82	54.98	3.73	100.66	

In Table 12 are given the results of experiments by Osborne et al. (36), Gortner (12), and Hart and Sure (20), showing the effects of added carbohydrates upon the nitrogen distribution of some proteins. An examination of these data shows that in all cases the presence of carbohydrate materially increases the humin nitrogen. The ammonia is significantly affected, however, only when sucrose and starch are added. The presence of the pentosan, xylan, while increasing the humin nitrogen by 700 per cent, has no effect upon the ammonia fraction. This would indicate that the lowering of the ammonia, where it does occur, is not attributable to adsorption on the humin. In order to verify this conclusion, the following experiment was performed:

Two 5-gm. samples of wheat glutenin (the same preparation as analyzed for nitrogen distribution) were weighed out. To one, 2 gm. of cornstarch was added. To the other 1 c. c. of redistilled furfural was added. Each was hydrolyzed for 21 hours with 20 per cent HCl. The acid-insoluble humin was filtered off and washed until free of chlorine ion. The humin precipitates were then treated in the same manner as for the determination of ammonia nitrogen; that is, 150 c. c. water, 100 c. c. 95 per cent alcohol, and an excess of  $\text{Ca}(\text{OH})_2$  suspension were added to the precipitate in a Claisen flask. This was then distilled at low temperature under suction for 30 minutes. Not a trace of ammonia was obtained by this procedure. The acid-insoluble humin nitrogen increased from 0.52 per cent of the total nitrogen to 1.85 per cent where the starch was added, and to 2.05 per cent where the furfural was added. Evidently, then, the lower ammonia nitrogen observed when carbohydrates are present can not be accounted for by adsorption on the humin. There seems to be only one conclusion possible, namely, that the amide groups are simply not split off. But from a purely chemical viewpoint it seems unreasonable to believe that the presence of substances such as hexoses and pentoses would protect an acid-amide linkage against hydrolysis by 20 per cent HCl. From these facts it might be assumed that the amides are not the only source of ammonia during hydrolysis, but that some amino acids undergo partial deamination. That this actually occurs has been demonstrated by Gortner and Holm (15, 16). Accordingly, it is conceivable that the presence of a large amount of aldehyde, derived from the sugars, might affect the rate of deamination, or the aldehydes, acting as oxidizing agents, might remove one or both hydrogen atoms from these amino groups, with the result that cyclic derivations of the pyrole and pyridine type would result.

Gortner and Holm (16) have shown that deamination occurs when pure amino acids are boiled with 20 per cent hydrochloric acid, but that when tryptophane and aldehyde are present there is an increase in the humin nitrogen and a decrease in the ammonia nitrogen. Tryptophane is relatively easily deaminized by boiling hydrochloric acid in the absence of aldehydes, but the humin resulting from interaction of tryptophane and aldehyde is very resistant to acid boiling (14). Gortner (12) has also shown that when formaldehyde is present in a fibrin hydrolysis "the ammonia nitrogen falls significantly when quantities of formaldehyde are added, but rises very rapidly when larger quantities are present."

Roxas (40) suggests the formation of cyclic nitrogen compounds as contributing to the humin nitrogen values. While undoubtedly the true humin nitrogen is largely or wholly derived from tryptophane, such compounds as were postulated by Roxas may be responsible for the formation of a part of the humin formed by hydrolysis in the presence of carbohydrates and may affect the ammonia fraction. However this may be, the liberation of ammonia nitrogen in a protein hydrolysate involves more reactions than simply the hydrolysis of amide linkages, and is one of the many things demanding further investigation. In view of the foregoing considerations, one would not be justified in attaching very great value to the ammonia fraction in comparing proteins which contain any great amount of impurity, unless the nature of the impurities was specifically known.

Turning next to the basic fractions, it is seen that the effect of the presence of carbohydrates is in most instances relatively slight. The results for arginine are somewhat anomalous. Hart and Sure obtained a slight decrease with glucose, while Osborne reports a slight increase. This probably indicates that there is little significant effect. In all of the other cases, however, there is a decrease of arginine nitrogen which can not be ignored. Moreover, the total basic fraction shows a decrease. This can scarcely be explained as due to the decomposition of some of the basic amino acids during hydrolysis, since in that case one would expect an increase of the ammonia fraction. This is not the case. Moreover, Gortner and Holm (15) have shown that arginine, histidine, and lysine are very stable to the action of boiling hydrochloric acid and undergo no appreciable deamination even when boiled with hydrochloric acid for six weeks. Neither do the data of Table 12 justify the assumption that the bases are adsorbed on the humin. There is practically equal lowering of total basic nitrogen in the case of starch and of xylan, but the difference in the humin, judged from the values for humin nitrogen, is very great. One other explanation is possible. The phosphotungstates of the bases have a tendency to exist in the colloidal state as a gel. Carbohydrates present might act in the rôle of protectors, preventing the crystallization of the phosphotungstates. If this were the case, it could be predicted that great irregularity would result in the precipitation, under these conditions, of a mixture of amino acid phosphotungstates, since these would all be affected differently by different carbohydrates. An experiment to test this assumption could be carried out readily by treating various aliquots of a known hydrolysate from which humin and ammonia had been removed in the usual manner, with varying quantities of different carbohydrates and then precipitating the bases with phosphotungstic acid. Or it would be better still to start with a known solution of arginine, histidine, and lysine salts.

Effects upon the ammonia, humin, and basic fractions are reflected in the filtrate fraction. Failure of arginine, histidine, and lysine to precipitate will result in an increase of the nonamino nitrogen of the filtrate. This, in general, is what happens, but the increase of the nonamino nitrogen fraction is by far too great to be accounted for in this way. There seems to be no explanation for these data, especially in view of the anomalies presented in the case where xylan is present. There the nonamino filtrate nitrogen drops to less than one-half the normal figure. Does this mean that the prolines were

adsorbed on the humin? How is this to be reconciled with the preceding data? Assuming that these analyses are correct, it appears to be utterly impossible to adduce any explanation which would fit all the facts, but from all the data presented in Table 12, there may be drawn the general conclusion that the ammonia, and the various basic fractions are least affected by the presence of carbohydrate impurities, while the humin and monoamino fractions may be profoundly changed.

The data just discussed, with the exception of the lactalbumin, have all been drawn from mixtures in which the carbohydrate has been very high, two to five times greater than the amount of protein present. It seems not unreasonable to assume that with very much smaller amounts of carbohydrate present, the variations from the true values would be smaller. This would be true especially with regard to the basic fractions.

The foregoing considerations have led to the conclusion that in comparing proteins of doubtful purity, the humin and the filtrate fractions are of least value. It is believed, however, that considerable reliance may be placed on the basic fractions. The ammonia nitrogen, while not without value, should not be relied upon as having great accuracy since it may or may not be affected, according to the sort of impurity present.

Before proceeding further it is desired again to call attention to the analyses of the three preparations from rye. The data shown in Table 6 confirm, to some extent, the foregoing conclusions regarding the effect of impurities.

The three preparations were analyzed to determine whether or not the proteins in each were identical. If this proved to be the case, the analyses would serve as an excellent means of judging the relative effect of the impurities present, since the preparations varied in nitrogen content from 13.67 to 15.61 per cent.

Examining the data, it is seen that there is wide variation in the ammonia nitrogen, even in the case of preparations 3 and 5, in which the total humin values are identical.

The arginine values and the total basic nitrogen values are remarkably constant. The cystine values are of the same order, and the variation is no greater than the experimental error involved in dealing with such minute quantities of material by gravimetric methods. The histidine values for preparations 2 and 3 agree well, but the value for preparation 5 is very much lower. This is considered not significant because of the high probability of error in the amino nitrogen determination. The same thing may be said of the non-amino nitrogen of the filtrate. It should not be inferred that the possibility of absolute accuracy with the Van Slyke apparatus is called into question. It is simply admitted that the technic used is not under as accurate control as would justify the use of these data in finally judging a protein. In the writer's opinion, however, there is presented sufficient evidence here to warrant the conclusion that the proteins of the three preparations are identical. On this assumption the data may now be examined to determine what effect the varying amounts of impurities have had upon the analyses.

Preparations 2 and 3 show rather regular differences. The high humin of 2 is accompanied by a corresponding lowering of the

ammonia fraction. Curiously, the sum of the amide nitrogen and the total humin nitrogen for both 2 and 3 is practically the same, being 15.07 and 15.10 per cent, respectively. Preparation 5, however, is irregular in comparison with the other two. The humin nitrogen is of the same value as that of 3, whereas the amide nitrogen is much lower, being in fact the lowest value for ammonia observed in the whole series of analyses. Since the basic fraction seems to have been, on the whole, relatively unaffected, wide differences necessarily show in the filtrate fraction. These anomalous results may be due to the extraordinarily high ash content, 19.16 per cent, of this preparation. The writer is utterly at a loss to account for this high ash content.

The consideration of these data, together with those of Table 12, seems to justify the conclusion that the arginine and the total basic nitrogen fractions may be used with safety in comparing proteins of doubtful purity. Standards for purity of protein after all are vague and for the most part very empirical.

TABLE 13.—Nitrogen distribution of some of the cereal glutelins, determined by the Van Slyke method

Source	Analysis by	Ammonia N		Nitrogen of bases					Nitrogen in filtrate from bases		Total recovery
		P. ct.	P. ct.	Arginine	Cystine	Histidine	Lysine	Total	Amino	Nonamino	
<i>T. vulgare</i> (patent).....	Blish (4).....	16.50	1.84	9.69	0.18	5.47	2.61	17.95	52.59	9.52	99.40
<i>T. vulgare</i> (biscuit).....	do.....	16.17	1.66	9.27	.18	7.59	1.90	18.94	53.38	9.35	99.50
<i>T. vulgare</i> (Idaho).....	Cross and Swain (8).....	15.62	1.95	10.10	.72	7.06	4.74	22.62	54.02	3.66	97.87
<i>T. vulgare</i> (patent).....	do.....	15.98	1.80	8.17	.71	9.22	4.93	23.03	54.57	3.81	99.19
<i>T. vulgare</i> (club).....	do.....	14.17	2.77	9.23	.65	11.42	4.72	26.02	56.55	2.64	102.15
<i>T. vulgare</i> (forty-fold).....	do.....	13.11	2.60	12.94	.65	6.20	6.42	26.21	55.62	3.98	101.52
<i>T. vulgare</i> (patent).....	Hoffman and Gortner (22).....	13.56	1.44	11.96	.67	4.72	4.52	21.87	53.47	7.50	97.84
Do.....	Larmour.....	14.78	1.70	10.90	4.0	1.67	5.83	18.80	58.15	5.39	98.82
<i>T. durum</i> .....	do.....	13.25	2.49	11.00	.88	5.84	3.48	21.20	54.47	9.01	100.42
<i>T. spelta</i> .....	do.....	8.06	5.02	13.43	.41	2.80	7.88	24.44	56.35	5.72	99.59
<i>T. dicoccum</i> .....	do.....	11.06	4.63	13.03	.75	4.80	6.02	24.60	54.41	4.93	99.63
<i>T. monococcum</i> .....	do.....	10.78	6.04	11.86	1.04	2.44	4.82	20.16	56.66	6.60	100.24
<i>Avena sativa</i> .....	do.....	11.87	3.53	15.48	1.10	3.23	4.56	24.37	57.72	2.22	99.71
Do.....	Luers and Siegert (27).....	12.19	2.89	14.43	1.52	7.24	4.39	27.58	52.35	4.08	99.09
<i>Zea mays</i> .....	Larmour.....	11.32	2.58	9.45	1.00	3.90	4.49	18.84	61.48	5.60	99.82
<i>Euchlaena mexicana</i> (teosinte).....	do.....	10.16	8.27	14.15	1.18	7.98	3.53	26.85	49.65	5.32	100.25
<i>Secale cereale</i> (2).....	do.....	9.78	5.29	13.65	.47	5.22	6.54	25.88	51.94	6.40	99.29
<i>Secale cereale</i> (3).....	do.....	10.80	4.30	13.86	.68	5.25	5.61	25.40	52.59	6.90	99.99
<i>Secale cereale</i> (5).....	do.....	7.14	4.30	13.60	.60	3.66	7.73	25.79	58.14	4.36	99.73
<i>Hordeum vulgare</i> .....	do.....	11.88	3.26	11.08	1.79	2.05	5.33	20.25	57.67	6.84	99.40
<i>Oryza sativa</i> .....	Kondo (25).....	12.36	.66	14.89	.14	4.55	5.94	25.52	58.75		97.29
Do.....	Osborne, Van Slyke, Leavenworth, and Vinograd (56).....	11.33	1.59	17.17	.46	4.77	4.82	27.22	52.98	6.08	99.20
Do.....	Larmour.....	8.07	1.95	17.95	.61	4.60	4.43	27.59	55.96	6.56	100.13

\* Total N in filtrate.

To facilitate comparison of the experimental data herein recorded with similar data of other investigators, all available analyses of cereal glutelins are shown in Table 13. No Van Slyke analysis of the glutelin of corn has been discovered. The analyses of glutenin by Cross and Swain have been included despite the fact that they are considered by the writer as of doubtful value.

Attention should be called to the fact that where the various fractions of the humin nitrogen have not been reported, in all probability the total humin nitrogen represents only the sum of the acid-insoluble and acid-soluble humin nitrogen. Therefore the values in total humin nitrogen will always be somewhat lower than those in which is included the phosphotungstic acid humin nitrogen.

A comparison of the writer's results on glutenin with those of Blish, shows great variations in the ammonia nitrogen, the histidine, and the lysine fractions, and in the two filtrate fractions. There is fairly close agreement, however, in the values for arginine, total basic nitrogen, and total nitrogen of the filtrate from the bases. This indicates that there have been differences in the manipulation of the amino nitrogen determination.

Turning next to the analyses of avenin from oats, even greater differences are noticed. The ammonia nitrogen in this case checks very well, as does also the humin nitrogen, if allowance is made for the phosphotungstic acid humin fraction. The arginine nitrogen is higher than that of Lüers and Siegert, while the total basic nitrogen is lower. This, of course, throws the histidine values out of agreement. It is a surprising fact that the lysine values agree. It must be admitted that, on the whole, there is not very good agreement between these two determinations. There might be offered in explanation the fact that the source of the two preparations varied widely, since one was grown in Germany and the other in America. Not much value is attached to this explanation, however.

More satisfactory results were obtained with oryzenin. There is very close agreement with the analysis by Osborne, Van Slyke, Leavenworth, and Vinograd, except for the ammonia nitrogen and the amino nitrogen of the filtrate from the bases. The humin, arginine, cystine, histidine, lysine, and nonamino nitrogen of the filtrate fractions all agree as closely as could be expected for different preparations of the same protein by different workers. The ammonia nitrogen of the writer's analysis is unaccountably low and there is a corresponding increase in the amino nitrogen of the filtrate from the bases. The agreement with Kondo's data is not as good.

These comparisons furnish further evidence for the conclusion previously expressed that the basic fractions should be considered the most reproducible and reliable values in the whole analysis.

In making a comparison of the analyses of all the proteins it would be well to bear in mind the fact that up to the present glutenin, oryzenin, and corn glutelin have been considered the only well-defined representatives of the glutelin class. These then should be useful in adjudging the relationship of the others.

On examining these data one must be led to conclude that there is a very wide range in the degree of purity. The humin fraction varies from 1.70 per cent for glutenin to 8.27 per cent for the glutelin from teosinte. Correspondingly, the nitrogen content of the preparations varies from 16.64 per cent for glutenin to 11.02 per cent for teosinte. The same treatment that was necessary to produce a preparation of wheat glutenin and of oryzenin comparable in purity with those obtained by other workers, failed to give more than a very impure product in the case of most of the other cereal glutelins. This is due doubtless to the presence of gums. Methods more

refined than those in use at present will have to be devised before glutelin preparations of a high degree of purity can be obtained.

The evident impurity of many of the preparations makes it impossible to draw comparisons between the humin values. The same objection applies to the total nitrogen of the filtrate from the bases. The ammonia values may have some significance, but for reasons previously given little reliance can be placed on them. Since the nature of the impurity present in the protein is unknown, one is unable to say what effect it might have had on this fraction. However, the ammonia nitrogen values for all the new proteins fall within the range of values for glutenin and oryzenin as shown by the rearrangement in Table 14.

TABLE 14.—*Values of ammonia nitrogen of the various glutelins, arranged in order of magnitude*

Glutelin	Ammonia N	Glutelin	Ammonia N
	<i>Per cent</i>		<i>Per cent</i>
Glutenin.....	<sup>a</sup> 16.50	Dicocco-glutenin.....	11.06
Duro-glutenin.....	14.78	Monococco-glutenin.....	10.78
Avenin.....	13.25	Teozeanin.....	10.16
Hordenin.....	11.87	Secalenin (average).....	9.24
Zeanin.....	11.38	Oryzenin.....	8.07
	11.32	Spelta-glutenin.....	8.06

<sup>a</sup> Blish, (4).

TABLE 15.—*Values of arginine nitrogen of the various glutelins arranged in order of magnitude, with corresponding values of total basic nitrogen*

Glutelin	Arginine N	Total basic N	Glutelin	Arginine N	Total basic N
	<i>Per cent</i>	<i>Per cent</i>		<i>Per cent</i>	<i>Per cent</i>
Glutenin (Blish (4)).....	9.27	18.94	Dicocco-glutenin.....	13.03	24.60
Zeanin.....	9.45	18.84	Spelta-glutenin.....	13.43	24.44
Glutenin (Larmour).....	10.90	18.80	Secalenin (average).....	13.77	25.69
Duro-glutenin.....	11.00	21.20	Teozeanin.....	14.15	26.84
Hordenin.....	11.08	20.25	Avenin.....	15.48	24.37
Monococco-glutenin.....	11.86	20.16	Oryzenin.....	17.95	27.50

The same thing holds true for the arginine and total basic nitrogen values, although the order is somewhat different from that shown above. Table 15 gives the arginine nitrogen values arranged in order of magnitude and the corresponding values for total basic nitrogen. The latter values, it is seen, fall in the order of the arginine except for monococco-glutenin, the difference between it and hordenin being negligibly small, and avenin, which is three places out.

Summarizing the data, it is seen that glutenin and oryzenin, the best known glutelins, appear to occupy practically the extreme opposite limits of the class in respect to ammonia nitrogen, arginine nitrogen, and total basic nitrogen. Corresponding values for all the other cereal glutelins fall between these limits in all cases. Consequently, if the assumption regarding the reliability of the arginine and total basic values is correct, it must be concluded that all these proteins under discussion possess enough common chemical characteristics to warrant their inclusion in the class with glutenin and

oryzenin. Therefore the writer feels justified in stating that the class of proteins known as glutelins is definite in character, and that proteins of this type exist in all of the cereal grains thus far investigated.

#### SUMMARY

Alkali-soluble proteins have been prepared from the cereal grains, *Triticum vulgare*, *T. durum*, *T. spelta*, *T. dicoccum*, *T. monococcum*, *Avena sativa*, *Hordeum vulgare*, *Secale cereale*, *Zea mays*, *Euchlaena mexicana* (teosinte), and *Oryza sativa*.

Analyses for nitrogen distribution by the Van Slyke method revealed a well-marked relationship between the various preparations, especially in respect to the basic nitrogen fraction.

Glutenin of wheat, *Triticum vulgare*, and oryzenin of rice, *Oryza sativa*, both well-defined glutelins, occupy positions at the extreme opposite limits of the class in respect to ammonia nitrogen (practically) and total basic nitrogen, and the corresponding values for the other proteins described fall within these limits. This is submitted as evidence that the preparations obtained belong to a definite class of proteins, the glutelins, which is represented in all the cereal grains thus far studied.

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# VANILLA ROOT ROT<sup>1</sup>

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## THE HOST

The vanilla of commerce (*Vanilla vanilla* (L.) Br., *V. planifolia* Andr.) is cultivated principally in Mexico, Guadeloupe, Venezuela, Tahiti, and Moorea in the Western Hemisphere, and in Madagascar and its dependencies, Ceylon, Mauritius, Reunion, and Seychelles Islands, and Java, in the Eastern Hemisphere. The plant is native to Central America. The cultivation of vanilla on a commercial scale in Porto Rico was begun in 1909 by McClelland (7),<sup>2</sup> who demonstrated the possibility of profitable production in that island.

The vanilla climbs by means of "holdfasts," that is, short, slender, rootlike organs arising from the nodes. The holdfasts flatten on the side which comes in contact with a supporting surface and attach themselves to it by means of numerous minute haustoria-like projections. The projections do not behave as haustoria and absorb nutrients from the supporting plant, for they do not penetrate the bark to the cambial tissue. Probably the only function of the holdfasts is that of attachment and support. Plants growing upon dead wood or upon inorganic supports thrive equally with those growing upon living trees. In neither case, however, does the plant grow if its connection with the soil is severed. These conditions are, therefore, seen to be at variance with the statement of Cook and Collins (4) that "vanilla is in reality merely an epiphyte or air-plant able to elaborate its food directly from the atmosphere," and also of Newport (12) that "the greater part of its nutriment is obtained from the atmosphere by means of its thick, fleshy leaves and tendrils or aerial roots."

The roots arise from the nodes. When a cutting is planted with some of the nodes covered with earth or organic matter, the first roots appear at the covered nodes and the later roots at the exposed nodes. The young roots are very succulent and easily broken. They attach themselves to any available body, elongate rapidly, and exhibit positive geotropism, the tip continuing its downward growth. The holdfasts may be a modified form of root, since they are similar to the roots in habit of attachment and occasionally are transformed into them upon coming in contact with moist, decaying organic matter. A young root which fails to become attached to some surface remains an aerial organ until it reaches the ground and enters the soil. Root hairs, giving the root a white distinctly hairy appearance, grow in abundance where the root is in contact with the soil. The roots

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<sup>2</sup> Reference is made by number (italic) to "Literature cited," p. 1135.

branch profusely, and the branches are succulent. The roots do not penetrate the soil deeply, and may not enter a heavy clay soil having a moisture-retaining mulch of decaying organic matter. The number of roots produced by a single plant varies greatly with different conditions. One or two roots may arise from a node, and the number of nodes producing roots depends upon the age of the plant and the availability of plant nutrients. Few roots are necessary to supply the needs of a plant grown in a moist, porous soil which is free from disease and well mulched with decaying organic matter. Roots are borne in profusion, however, when the plant is grown under adverse conditions, including lack of moisture, scarcity of organic matter, and a compact, inhospitable soil, or when the established roots have been destroyed.

Like most orchids, vanilla is a shade-tolerant plant and grows in the shade of other plants, which serve the double purpose of screening it from the rays of the sun and supporting the vines. In Porto Rico the dwarf bucare (*Erythrina berteroana*) has been found practicable for carrying vanilla. The blossoms must be hand-pollinated, and the first crop may be obtained in three or four years after planting.

## THE DISEASE

### HISTORY

A root disease of vanilla was first noticed in Porto Rico in 1918 in the vanillery at the station in Mayaguez. In a short time the planting became so severely infected that it failed to produce a crop, and eventually the plants died. The earliest reference to the disease is that of Thomas (17), who isolated a species of *Fusarium* from diseased roots. What is most likely the same fungus is listed by Stevenson (16) as *F. solani* (Mort.) Sacc. McClelland (8) in 1922 recorded considerable damage to vanilla by "a root disease, presumably *Fusarium* sp." In the same year Bregger (3) noted the occurrence of a root disease which did considerable damage to *V. planifolia*. McClelland (9) in 1923 reported that "experimental work under way with vanilla had to be abandoned due to a root disease, presumably *Fusarium* sp." Progress reports on the work herein described were published by the writer (18, 19) in 1924 and 1926. McClelland (10) in 1924 stated that growers of vanilla "must consider it a temporary crop because of this root disease."

In the Seychelles Islands, where vanilla was formerly an important crop, the same or a very similar root disease is present, according to a report made in 1921 by Dupont (5). In describing the disease he says:

It seems to me that the roots are first affected. As soon as they reach the ground their tips die off and this die-back eventually kills the whole root system. Aerial roots are next formed in succession to replace the dead roots and it seems on theoretical grounds that the whole energy of the plant is exhausted in producing these organs. As many as three aerial roots have been found to be produced in succession from the same node.

The disease as described above coincides quite closely with that occurring in Porto Rico.

In 1925 Van Hall (6) mentioned the occurrence in Java of a root fungus which caused the death of cuttings.

The references cited unquestionably indicate the severity of the disease, and the need for safeguarding the vanilla industry in Porto Rico.

#### SYMPTOMS

The earliest visible symptom of the disease is the browning and death of the roots. Since many of the roots are in part aerial, their death is rather conspicuous. The underground portion of an infected root dies first. It turns dark brown and decays, and the rot is either soft and watery or somewhat dry, depending upon existing moisture conditions. The progress of the rot is checked at the surface of the soil, and often roots which are apparently healthy will be found to have rotted beneath the surface. The aerial portion of the root slowly shrivels and dies, but it may retain its normal green color for several months after the death of the lower portion. Dying back as the direct result of fungus invasion does not occur, and branches sometimes arise from the aerial portion above an infected region. The branches descend into infected soil and quickly become diseased. Branches springing from infected roots when placed in uninfected soil by the writer made normal growth and were free from disease, indicating that the disease does not always become systemic in the root tissues. Frequently, where soil infection is established, the aerial roots descending from supporting trees are killed before reaching the ground.

With the destruction of the roots the plant ceases growth and devotes its energy to proliferating roots in an attempt to reestablish connections with its food supply. The tender growing tip dies and falls away, the stems and the leaves become a sickly yellow, and the stems shrivel as their reserves of nutrients are removed. The efforts to reestablish roots may continue for a year or longer until the plant exhausts itself and dies. Some diseased plants are enabled to live several years due to their descending roots, which enter the soil and live for a short time before they become infected. Roots 15 feet long have been seen descending the trunks of supporting trees. The older portion of the vine usually dies as it exhausts its capacity for root proliferation. In some instances as much as the lower 10 feet of a vine may rot away, whereas the upper part is still green and continues its precarious existence on the small amount of food obtained through the diseased roots. Recovery is unknown and the length of time from infection to death is dependent upon the size and vigor of the plant and the severity of the infection.

So far as is known, none of the few plantings in Porto Rico is free from the disease. The losses caused by it depend principally upon the age at which the plants become infected. Early infection may cause the death of the plants prior to bearing.

#### THE CAUSAL ORGANISM

Since beginning the study of the vanilla root disease in 1923 the writer has made several hundred isolations from infected roots. In about 90 per cent of the isolations a *Fusarium* has been the only organism obtained. The strains taken from different plantings at different seasons of the year were so nearly identical that the same

fungus was regarded as the causal organism. This premise, as will be shown, has been substantiated by inoculations made under controlled conditions.

The *Fusarium* grows well on a large range of culture media. Steamed rice is the most satisfactory medium for isolating the fungus. Sections of diseased roots, including the margin of the rotted area, were surface-sterilized in a solution made by dissolving 1 gm. of mercury chloride in 500 c. c. of 50 per cent alcohol. They were then washed in sterile water, split open, and placed in tubes of steamed rice. A sterile rod was used to push the sections into the rice. After three or four days a white growth of mycelium was observed arising from the root sections. On rice the initial growth of mycelium is white, and the substratum becomes colored as the age of the culture advances. In 4-weeks-old cultures the color of the substratum varies from white in the most recently invaded sections to light pink and Corinthian pink (Ridgway's Color Standards) (14) in the older portions. Microspores are borne very abundantly, but macrospores are less in evidence. At four weeks the macroconidia present were mostly one-septate, and 9.2 to 17.5 microns long, with an average length of 13.5 microns. Two-septate spores were 16.7 to 20 microns long, with an average length of 17.8 microns. Three-septate spores were 26.7 to 35 microns long, with an average length of 31.6 microns. As the cultures age the rice gradually changes from reddish to deep blue. A strongly aromatic odor is present in all rice cultures.

In order to obtain data on the morphology and physiology of the fungus for comparison with other species for purposes of identification, suggestions of the Wollenweber, Sherbakoff, and their associates (21) were followed and, with the exception of stems of *Melilotus*, *Lupinus*, and *Alnus*, which were not available, the media recommended by them were used for the cultivation of the vanilla *Fusarium*.

On potato tuber cylinders the fungus produced an abundant white, cottony growth in three weeks. Bluish sclerotia were sparingly produced. They were of irregular shape and composed of a compact mass of bluish-tinged hyphae and chlamydospores. Microconidia and chlamydospores were borne abundantly, and only few macroconidia appeared. The chlamydospores were borne in chains, or singly, intercalarily, or terminally on short branches, and varied in diameter from 6.4 to 10.4 microns, with an average diameter of about 8.6 microns. The microspores were cylindrical, guttulate and hyaline, and varied in length from 4.8 to 6.4 microns, and in diameter from 2.4 to 3.2 microns. The mycelium varied in diameter from 1.6 to 6.4 microns, the former young and hyaline, and the latter granular, closely septate, and forming chlamydospores. There was no discoloration of the substratum.

On oatmeal agar, made as recommended by Sherbakoff (15), sporodochia were borne in 2-weeks-old cultures. The sporodochia varied in color from white to lilac, those of deeper color being produced near the bottom of the slope. Macroconidia were abundant, nearly always three septate, and varied in length from 28.4 to 42.6 microns. Microconidia also were produced, but chlamydospores were not seen.

On potato-dextrose agar containing 2 per cent of dextrose 2-weeks-old cultures produced numerous white to purple sporodochia. Re-

cently isolated strains caused the agar to redden throughout. This character does not appear in long (2 years) isolated strains. Three-septate macroconidia were abundant. They were densely granular, with a tendency toward constriction at the septae, less distinctly pedicellate and less curved than the normal, and varied in length from 23.4 to 45.9 microns.

On potato-dextrose agar containing 5 per cent of dextrose, numerous deep reddish-purple sporodochia were present. Recently isolated strains cause the medium to turn reddish purple. In long isolated strain cultures the discoloration is confined to the sporodochia-bearing region. Macroconidia were abundant and often many 1 and 2 septate spores were present. The tendency toward constriction of the septae noted in potato-dextrose agar cultures containing 2 per cent of dextrose was intensified, and the normal curvature had disappeared. The macroconidia were blunt and rounded at the ends and certainly abnormal. They varied in length from 15.8 to 36.7 microns.

On *Crotalaria juncea* stems there was abundant white growth in two weeks. No color was observed. Macroconidia were abundant, mostly three septate, and apparently normal in size and shape. They varied in length from 21 to 45.1 microns. Microconidia also were abundant.

Microscopic examination of diseased roots revealed the presence of hyphae in the margins of the diseased and decaying portions, and spore forms in the older portions. The fluctuation in length and septation of macroconidia from inoculated rotting roots from tubes was as follows: 1 septate (very few), 13 to 17 microns; 2 septate (very few), 22 to 32 microns; 3 septate (very numerous), 23 to 55 microns; 4 septate (very few), 32 to 58 microns; and 5 septate (one seen), 60 microns. The macroconidia were hyaline, curved, pedicellate, and somewhat attenuated at the apex, and not constricted at the septae. Dry decaying roots yield chlamydo-spores occurring singly or in chains of two to four. (Fig. 1.)

Averna-Saccá (1), in a morphological description of an unnamed *Fusarium* which attacks vanilla in Brazil, described the macrospores as—

fusiformes, com ápices arredondados, rectos ou ligeiramente curvados, hyalinos, (21.6 a 43.2 por 3 a 5.5 microns), primeiro continuos, depois 1-3-4-septados. Em geral, 3 septados.

From the description, the fungus resembles quite closely that in Porto Rico. Averna-Saccá (1), however, stated that his *Fusarium* attacks the leaves and stems, causing small, light, irregular spots, and he did not mention its occurrence on the roots.

Meinecke (11) observed an undescribed *Fusarium* attacking the tender tips and young pods of vanilla vines in Tahiti and Moorea, with the formation of small pustules. He did not find it as a root parasite.

The characters of the macrospores place the vanilla parasite in the *Elegans* group. In attempting to place the fungus in a previously described species, *Fusarium cubense* EFS. was immediately suggested because of the great preponderance of three septate spores the aromatic odor on steamed rice, and the almost universal infection of

soils in Porto Rico by *F. cubense*. The chlamydospores of the vanilla fungus are somewhat larger than those of *F. cubense* and salmon-colored masses of macroconidia have not been observed in

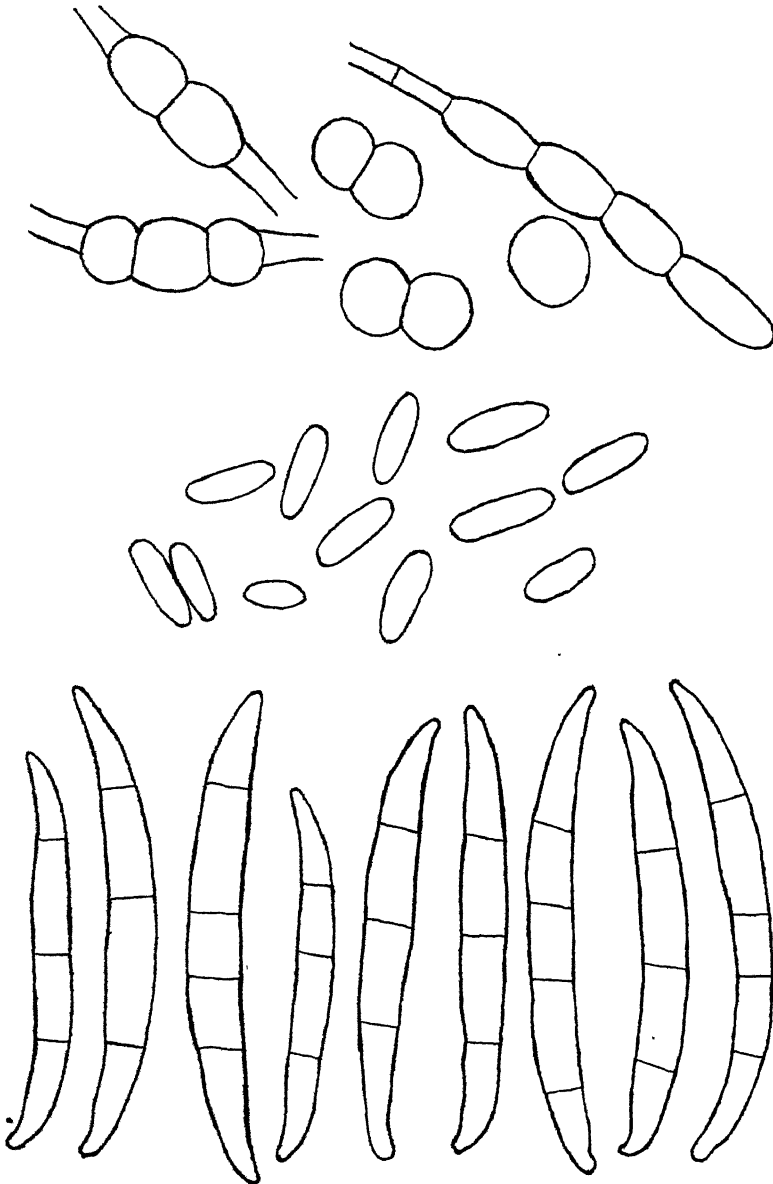


FIG. 1.—*Fusarium batatatis* var. *vanillae* n. var. Macroconidia, microconidia, and chlamydospores in various stages of development.  $\times 1,590$

the former, perhaps because of the profuse white mycelial growth commonly obtained. Results of inoculation experiments prove that *F. cubense* is not pathogenic to vanilla.

A culture of the vanilla fungus was sent to C. D. Sherbakoff, who considered it near to *Fusarium batatatis* Wollenw. (26). However, subsequent inoculations of sweet potatoes failed to produce infection, and a culture of *F. batatatis* received from L. L. Harter failed to cause infection of vanilla. The *Fusaria* resemble each other quite closely morphologically, and differ principally in the type of sclerotia produced. Furthermore, the vanilla *Fusarium* produces sclerotia much less abundantly than does *F. batatatis*. The fungi exhibit marked differences in pathogenicity. The vanilla fungus is considered to be *F. batatatis* var. *vanillae* n. var.

***Fusarium batatatis* var. *vanillae*, n. var.**

Sporodochia borne on decaying vanilla roots. Macroconidia, usually 3 septate, occasionally 1 to 2 septate, rarely 4 to 5 septate. Three-septate spores, 23 to 45 by 2.6 to 4 microns, with an average of 34.2 by 3.6 microns. Macroconidia curved, pedicellate, hyaline, not constricted at septae; apical cell somewhat attenuated. Chlamydospores thick-walled when old, single or in short chains, brown, 6.5 to 10 microns. Microconidia hyaline, oval-elongate, not borne in chains, 4.5 to 7 by 2.2 to 3.6 microns. Blue irregular sclerotia on potato cylinders. In steamed rice cultures the substratum turns vinous red and finally blue. Potato-dextrose agar is reddened, a character which is lost in long-isolated strains. Reddish-purple sporodochia produced on oatmeal and potato-dextrose agar.

Differs from *Fusarium batatatis* Wollenw. in its failure to produce blisterlike sclerotia, which are pushed through the thallus covering the substratum. *F. batatatis* produces sclerotia more abundantly. *F. batatatis* is pathogenic to sweet potatoes and nonpathogenic to vanilla; *F. batatatis* var. *vanillae* is nonpathogenic to sweet potatoes and pathogenic to vanilla.

Parasitic on roots of *Vanilla vanilla* (L.) Br. in Porto Rico.

The pathogenicity of the fungus is due to a cytolytic enzyme which it produces, causing the dissolution of the parenchyma tissue of the root. The vascular system is resistant, and cross sections of diseased roots show browning and cytolyzing of the cell walls of the parenchymatous tissues, whereas the cells of the vascular tissues are yet uninvaded. Attempts to isolate the fungus from root sections taken 1 inch above the decaying area have always been unsuccessful. Of course the vascular region is finally invaded and destroyed, but there is never any evidence of the plugging of vessels as in the case of disease caused by *Fusarium vasinfectum* Atk. That there is no general invasion of the root is further indicated by the fact that some diseased roots which are killed back several inches above the soil bear a single branch arising at not more than an inch above the rotted portion. (Fig. 2.) The branch root descends to the soil and quickly becomes infected. In six cases where the writer placed such branches in sterile soil four roots grew normally. In the other two cases, the old root continued to decay and the young root succumbed when the disease reached the point at which the branch arose.

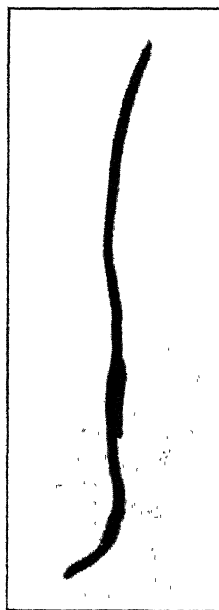


FIG. 2.—Branching of infected aerial root. The branch, if placed in a tube of autoclaved soil, would be likely to develop free from infection.

The above facts seem to preclude the possibility that the vanilla *Fusarium* produces a substance toxic to the plant as does *F. cubense* as shown in the experiments of Brandes (2). Brandes observed that cut banana plants wilted when placed in filtrates from cultures in Richards's solution and Uschinsky's solution. His experiments were closely followed by the writer to determine whether the vanilla *Fusarium* resembled *F. cubense* in this respect. Cultures of *F. cubense* and the vanilla fungus were grown in each solution for two weeks. The fungus was filtered out and cut-bean and cotton seedlings were placed in the filtrates. The results differed somewhat from those obtained by Brandes (2), since the wilting occurred in the sterile Richards's solution as well as in the culture filtrates. In Uschinsky's solution Brandes's results were duplicated by the wilting of the seedlings in the filtrates from cultures of *F. cubense*. The filtrate from cultures of the vanilla *Fusarium* failed to cause wilting, however, even after 18 hours. A study of the symptoms of the disease indicates that absence of a toxic product is to be expected. There is no wilting of the plant beginning with the older leaves as in the case of the banana. The slow dying accompanied by shrinkage of the succulent stems and general yellowing of the entire plant indicates death by starvation.

#### PROOF OF PATHOGENICITY

In order to determine whether the soil was a source of infection, twenty 8-inch pots were filled with soil from a vanillery in which most of the plants had died from root disease. Ten of the pots were autoclaved. One vanilla cutting was planted in each pot, and the pots were placed side by side under partial shade. Six months later the cuttings in the autoclaved pots were making healthy growth, and examination of their roots disclosed no disease. The cuttings in the pots of unsterilized soil made little growth, were yellow, and offered a striking contrast to the plants in the autoclaved pots. All the unsterilized pots contained diseased roots.

In the above-mentioned and in the following experiments, the vanilla cuttings were often taken from diseased vines. No evidence has been found to warrant the view that the disease is transmitted by cuttings except where possibly spores adhere to the surface. To eliminate this factor as a source of infection, all cuttings were dipped for five minutes in a 4:4:50 solution of Bordeaux mixture. This solution kills the tender tips of the plant, but does no serious damage since buds arise readily from the axils of the leaves. Most of the cuttings were 3 to 4 feet long. The supporting stakes were about 5 feet long and, like the cuttings, were dipped in Bordeaux mixture for the further prevention of infection.

In July, 1923, 18 cuttings were planted in autoclaved pots containing coconut fiber. Growth had started on each cutting by September 8, 1923, and the coconut fiber in 12 pots was inoculated with *Fusarium* grown for one month on moist corn meal. The fungus produced a copious growth and the contents of a liter flask was divided between two pots. The inoculum was mixed with the coconut fiber, care being taken not to injure the vanilla roots. Pots and plants were covered with cheesecloth to prevent the passage of insects acting as carriers.

Monthly observations were made and measurements of the new growth from each cutting were obtained. Table 1 gives the results of the experiment on September 18, 1924.

TABLE 1.—Results of inoculating vanilla growing in autoclaved coconut fiber with *Fusarium batatatis* var. *vanillae*

Treatment	Total number of plants	Number of diseased plants	Number of healthy plants	Average new growth of diseased plant	Average new growth of healthy plant	Average length of time to appearance of symptoms
Inoculated.....	12	7	5	Inches 54	Inches 109	Months 21½
Control (not treated).....	6	3	3	51	94	7½

Table 1 gives little evidence as to the pathogenicity of the organism used for inoculation, since the proportion of diseased plants among the controls is almost as large as among the inoculated. However, the average length of time ensuing between inoculation and the appearance of diseased roots is not without significance, since it varies from two and one-half months in the inoculated plants to seven and one-fifth months in the control plants. The table is of interest chiefly in showing the decreased growth in the diseased plants. The difference is much more marked in the inoculated group on account of the later infections occurring in the control group.

On October 2, 1923, 30 glass tubes, each 26 cm. long and 3½ cm. in diameter, were almost filled with moist coconut fiber, plugged with cotton, and sterilized. Ten tubes were inoculated by mixing corn-meal cultures of the *Fusarium* with the sterile fiber. In another 10 tubes 12 short sections of diseased vanilla roots were placed on top of the fiber. In a third series, 10 tubes were left to serve as controls. A surface-sterilized vanilla cutting was introduced into each tube so that the basal node would be in contact with the fiber. The tubes were then placed in a frame which held them upright and provided a support for the cuttings. The cuttings rooted quickly from the node in contact with the fiber. They were watered periodically, sterile water being used. The final notes were taken about 13½ months (November 14, 1924) after planting. Unfortunately most of the tops were cut off by a vandal and the data are therefore restricted to root growth and infection.

The accidental infections occurring in the experiment summarized in Table 1 were prevented by growing the cuttings in plugged tubes, but the results obtained in the inoculated tubes were unsatisfactory. Seven of the ten tubes which were inoculated with *Fusarium* from corn-meal cultures produced diseased roots, but the progress of the disease in the tubes was very different from that under field conditions. In the tubes single root branches became infected and decayed back a short distance, and new branches arose and often attained a length of 6 inches before becoming infected at the tips. In no case was the main root killed. Since only the basal 3 to 4 inches of the cutting were inside the tube, only one or in a few cases two roots entered the fiber from each cutting. The figures on root growth in

Table 2 represent the growth of branches, and the stimulation to branching due to the death of the small roots is shown by the increased root length of the diseased plants. The measurements represent the sums of the lengths of the root branches.

TABLE 2.—Results of inoculating vanilla in coconut fiber in plugged glass tubes

Treatment	Total number of plants	Number of diseased plants	Number of healthy plants	Average root growth of healthy plants	Average root growth of diseased plants
Inoculated with corn-meal cultures of <i>F. batatas</i> var. <i>vanillae</i> .....	10	7	3	Inches 163	Inches 191
Inoculated with pieces of diseased roots.....	10	2	8	125	147
Control (no treatment).....	10	0	10	159	-----

In casting about for an explanation of the unsatisfactory results recorded in Tables 1 and 2, an agar medium was prepared from an infusion of coconut fiber. Inoculations which were made with the vanilla *Fusarium* on the agar failed to produce growth. Inoculations of the infusion likewise failed to produce growth, although the fungus could be reisolated from the infusion. Coconut fiber is rich in tannin—a chemical inimical to the growth of fungi—and it is probable that the heating of the moist fiber during sterilization liberates sufficient tannin to inhibit the growth of the *Fusarium*. Since the foregoing inoculations were made in autoclaved coconut fiber, this inhibitory action is believed to account for the uncertainty of infection. Probably the concentration of the tannin was sufficiently high to prevent the growth of the fungus through the substratum and only those roots growing in direct contact with bits of the fungus-bearing corn meal became infected.

Three weeks after tubes of autoclaved moist garden soil were inoculated with the *Fusarium* the white mycelium of the fungus could be seen throughout. Garden soil was therefore used in all the experiments that followed.

In June, 1924, pots of autoclaved garden soil were planted with vanilla cuttings, one cutting per pot. Inoculations were made November 18, 1924, by stirring into the soil corn-meal cultures of *Fusarium* which were isolated from vanilla. The pots were partially shaded and were not covered with cheesecloth. Table 3 summarizes the results, which were obtained December 14, 1925.

TABLE 3.—Results of inoculating vanilla in garden soil

Treatment	Total number of plants	Number of—							
		Healthy plants after 3 months	Diseased plants after 3 months	Healthy plants after 4 months	Diseased plants after 4 months	Healthy plants after 6 months	Diseased plants after 6 months	Healthy plants after 12 months	Diseased plants after 12 months
Inoculated.....	50	26	24	24	26	12	38	0	50
Controls (no treatment).....	25	21	4	19	6	18	7	13	12

Six months after inoculation the number of aerial roots averaged 3.4 for diseased plants, and 1.8 for healthy plants. This furnishes experimental confirmation of increased root proliferation by diseased plants.

In the experiments, every inoculated plant became diseased, but the high percentage of diseased controls again lays the results open to criticism. Isolations from diseased roots from both inoculated and control plants gave a *Fusarium* which was apparently identical with that used for inoculation.

A method of inoculation was devised whereby the possibilities of accidental infections were reduced to a minimum. Tubes  $26 \times 3\frac{1}{2}$  cm. were two-thirds filled with moist soil, plugged with cotton, autoclaved, and inoculated with *Fusarium*. Healthy descending aerial roots 6 to 12 inches long were washed with 1:1,000 mercury chloride solution, rinsed with sterile water, and introduced into the inoculated tubes at the side of the cotton plug. The tubes were then tied to the support so as to keep the roots in as nearly their original position as possible. A tarred-paper cover was tied above the cotton plug and between the mouth of the tube and the support to prevent wetting of the plug by rain. (Fig. 3.)

Inoculations by this method were successful in every instance. Forty-six inoculations resulted in the death of the root, showing symptoms typical of natural infection. The *Fusarium* was reisolated from each root. Usually, the descending root is invaded and killed as it reaches the soil, and the length of time required for infection varies from 4 to 26 days. With very wet soil the root may come in contact

with the fungus on the walls of the tube and die before reaching the soil. (Figs. 4 and 5.) In any case, the roots rot very quickly in infected wet soil. In soil containing only a medium quantity



FIG. 3.—Method of inoculating aerial roots of vanilla in situ. The root is washed with mercuric chloride solution and inserted in the tube of autoclaved inoculated soil, which is tied to the support

of water the incubation period is longer. Twenty-five uninoculated control tubes supported normal root growth, the root penetrating to the bottom of the tube and bearing many branches. The control tubes were allowed to remain in place for two months and showed no evidence of infection.

Inoculations of slightly wounded roots always resulted in infection. (Fig. 6.)

A culture of *Sclerotium rolfsii* Sacc., isolated from vanilla roots at the Insular Experiment Station in Rio Piedras by M. T. Cook, was sent to the writer. Ten tubes were inoculated with the fungus and used for inoculating vanilla roots, but no infection was obtained.

To determine the possibility of infecting other portions of the vanilla plant, a water suspension of the *Fusarium* mycelium and spores was sprayed upon 10 vines. No infection

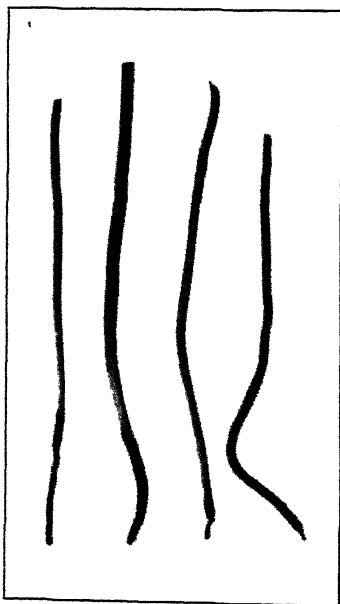


FIG. 4.—Unwounded vanilla roots from tubes of wet soil inoculated with *Fusarium batatas* var. *vanillae*. Photographed six days after the roots were placed in tubes

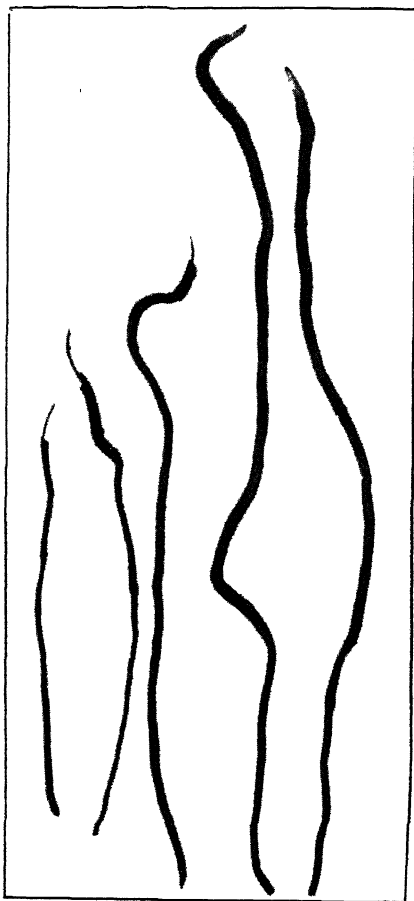


FIG. 5.—Left, two control roots from tubes of autoclaved soil; right, three infected roots from tubes of autoclaved soil inoculated with *Fusarium batatas* var. *vanillae*. Photographed 12 days after the roots were placed in tubes

resulted on leaves or stems. Ten stems were inoculated by placing the fungus in a shallow slit in the internodes. Seven days later a brown shrunk area surrounded by a dark green water-soaked margin was observed, and a viscous amber liquid exuded from the wounds. The invaded area enlarged, involving finally the whole internode, but in only two cases did the decay pass a node. The decay continued until

it completely destroyed the inoculated internode. (Fig. 7.) The fungus was reisolated from the margins of rotting internodes. A rotting of single internodes is occasionally seen in vanilleries, caused possibly by wound infection. This rot is distinct from the soft rot of the vines as described by Rangunathan (13).

Inoculations of wounded stems with *Fusarium cubense* produced no infections, and the wounds healed rapidly and were indistinguishable from those on 10 uninoculated control plants.



FIG. 6.—Three roots at the left were wound-inoculated with *Fusarium batatatis* var. *vanillae*; the roots at the right were wounded but not inoculated. Photographed five days after inoculation

The *Fusarium* is able to persist in the soil for at least four years. Cuttings that were planted in a vanillery in places where the plants had been killed by root disease four years previous were attacked and killed in less than a year. U tubes of sterile soil were inoculated in one arm. The average length of time required for the fungus to reach the surface of the soil in the uninoculated arm, a distance of 14 cm., was 10 days. In compact soil the rate of growth would probably be much slower, but the fact that the fungus can thrive in the soil when deprived of a living host indicates long retention of the organism by infected soils.

#### CONTROL

The problem of controlling vanilla root disease has some features in common with that of controlling banana wilt. Both are caused by a *Fusarium* which is able to persist in the soil for long periods. Both

hosts are ordinarily propagated by vegetative methods, which offer little opportunity for successful selection for resistance to the disease. Vanilla has the advantage of producing seed from which seedlings may be grown only with difficulty.

In addition to the valuable *Vanilla vanilla* (L.) Br., there are a number of species of Vanilla of low commercial value. One of these, known as the "pom-pom" type, is resistant to root disease. Plants of this vanilla when grown in an infected vanillery where all the *V. vanilla* was killed continued growth for 12 years. The plants are not immune to the disease, since many diseased roots may be found on them, but that they show a marked resistance is apparent from the luxuriant growth and dark green color.

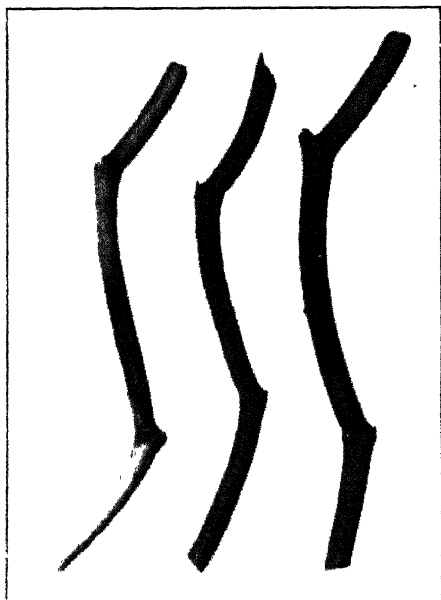


FIG. 7.—Sections of vanilla stems wound-inoculated with *Fusarium batatas* var. *vanillae*. Photographed three weeks after inoculation.

Crosses between *Vanilla vanilla* and the "pom-pom" vanilla offer a possible means of obtaining resistant strains of high commercial value.

Liming the soil to counteract acidity has been found to be valuable in combating banana wilt. Vanilla thrives best when feeding in a mulch of decaying organic matter. When such a mulch is maintained for several years, however, it is likely to produce a high acid condition, owing to the constant liberation of organic acids on a limited area. No data have been obtained on the effect of liming on vanilla.

McClelland (10) has recommended the planting of coffee with vanilla. Coffee thrives in locations that are favorable to vanilla, and the shade trees used as supports for vanilla furnish

the shade required for young coffee plants. Coffee is not attacked by the vanilla *Fusarium*, and vanilla is not susceptible to the coffee root fungi (*Rosellinia bunodes* and an undetermined basidiomycete). Under present conditions the vanilla may be expected to have passed its profitable age after six or eight years, at which time the coffee trees should be producing well.

In the inoculation experiments severe infection by *Fusarium* was observed to follow a disturbance of the soil or mulch in which the vanilla roots were growing. Vanilla roots are succulent and easily injured, and cultivation under field conditions should be avoided. The shade and mulch should be sufficient to prevent the growth of grass and weeds about the vanilla roots.

## SUMMARY

*Vanilla vanilla* (L.) Br., an orchid producing pods from which the vanilla of commerce is extracted, has been cultivated profitably in Porto Rico.

The roots of the plant are attacked by a root disease, caused by *Fusarium* sp., which is very severe and widespread. The fungus rots the roots, causing cessation of growth and the gradual death of the plant from exhaustion, due to the proliferation of new roots. These are killed upon reaching infected soil.

The *Fusarium* is a soil-inhabiting fungus and retains its activity in the soil at least four years and probably much longer.

Sterilized coconut fiber is an unfavorable medium for inoculation, probably because of the presence of tannin.

A method of inoculation of aerial roots in tubes of inoculated soil proved to be very satisfactory for demonstrating the pathogenicity of the fungus, and inoculations invariably resulted in typical infections from which the *Fusarium* was reisolated.

The vanilla *Fusarium* is described as a new variety, *Fusarium batatatis* var. *vanillae*. It also resembles *F. cubense* EFS. It is not pathogenic to sweet potatoes, and *F. cubense* and *F. batatatis* Wollenw. are not pathogenic to vanilla.

Crossing *Vanilla vanilla* with a less valuable but highly disease-resistant vanilla is suggested as a possible means of obtaining a disease-resistant strain of high commercial value.

Injury to the roots by cultivation is prejudicial, and mulch and shade should be maintained to prevent the growth of weeds and the necessity for cultivation.

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# CORRELATION OF KERNEL TEXTURE, TEST WEIGHT PER BUSHEL, AND PROTEIN CONTENT OF HARD RED SPRING WHEAT<sup>1</sup>

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## COMMERCIAL IMPORTANCE OF FACTORS DENOTING QUALITY IN WHEAT

Certain factors in wheat which are now recognized as indicators of milling and baking qualities are becoming increasingly important in determining the market price of wheat. These include grade, kernel texture, test weight per bushel, locality of production, and protein content. Because of the simultaneous occurrence of variations among these factors, it is difficult to compare the effect of each on price. Furthermore, their relative importance changes with the supply of and demand for wheat of the various types and qualities.

Before the development of modern transportation facilities and specialized occupations, when each community produced and prepared for consumption its own food supplies, differences in milling and baking qualities of wheat were of little or no commercial importance. The milling of wheat was then chiefly on a grist-grinding basis. As transportation facilities improved and occupations became more specialized, the grist-grinding trade decreased and the merchandising of wheat and flour increased. This increase in merchandising naturally led to a greater appreciation of the differences in the milling and baking qualities of wheat.

Undoubtedly, flour-yielding capacity was the first factor to be given attention. The plumpness of the wheat kernel was early associated with flour yield, and no doubt the miller was not long in learning that weight per bushel was a good index to plumpness, and, consequently, to flour yield. With the further development of transportation facilities which increased the sources of supplies of raw material and the possibilities for the extension of trade over wider areas, competition between mills increased. Coincident with the increase in competition, baking quality became of greater importance.

At about this time agencies were set up for the inspection or grading of wheat in order to facilitate the trading in that commodity. Inspectors, in accordance with certain rules, determined the grade, which was supposed to be an indication of the condition and milling value of the wheat. This was the beginning of the modern system of grain grading. The rules or specifications for these first grades were indefinite and left much to individual judgment, which varied considerably among the different inspectors. Uniformity in grading was impossible under a system of this kind. To aid in eliminating this condition the trade demanded more definiteness in the grade specifications.

<sup>1</sup> Received for publication Aug. 17, 1927; issued January, 1928.

<sup>2</sup> Valuable advice and assistance on the statistical phase of this work were rendered by H. R. Tolley and Mordecai Ezekiel of this bureau.

From the first, test weight per bushel was recognized as an important factor in grading. It is probable that gluten quality and quantity also were early recognized as important, but because of the time involved in making a test and the lack of a method whereby uniformity of results among different operators could be assured, these factors were left out of the grade specifications. Kernel texture was used instead. This was accomplished in the hard red spring wheat grades by making a distinction between the "hard" and the "soft" wheat types, and in the hard red winter wheats by the use of the terms "light," "dark," and "medium" in connection with the numerical grade to denote the general color or kernel characteristics of the sample. When the Federal Government established standards for wheat in 1917 this factor was recognized as important in indicating commercial value and was incorporated in the standards.

In harmony with the policy pursued in connection with the other grading factors, definite limitations were placed on the meaning of the terms used to designate the differences in hardness. In the case of the hard red spring and hard red winter wheat classes this was done by establishing subclasses based on the percentage of dark, hard, and vitreous kernels present. This, although accepted by the trade at the time as the best practicable means of designating quality, has never been considered wholly satisfactory, and with the development of improved analytical technic and an increased appreciation of quality, other methods more accurate and definite have been adopted by the trade for determining quality. The protein test is one of these methods, and has become of such commercial importance that a large proportion of the hard red spring and hard red winter wheats are being marketed to-day on that basis in conjunction with grade. Because of this widespread use of protein content in the grading of wheat a study of any relationships existing between it and the other factors used in the grades is of the utmost importance.

The only grading factors associated to any appreciable extent with kernel structure and composition are kernel texture, test weight per bushel, and moisture content. These, therefore, are the only grading factors that have any significant relationship to protein content. It is with these relationships that this paper is directly concerned. Moisture content, however, is taken into account only to the extent of converting all protein data used to the uniform moisture-content basis of 13.5 per cent. For information as to the relationship of the moisture content of wheat to protein content the reader is referred to United States Department of Agriculture Miscellaneous Circular No. 28 (11).<sup>3</sup>

#### CONCLUSIONS OF PREVIOUS INVESTIGATORS

A number of investigators (2, 5, 6, 9, 15)<sup>4</sup> have found that a relationship exists between kernel texture and protein content of wheat. Mangels (8), although admitting this relationship, concludes that the percentage of dark kernels can not be considered a reliable index of protein content for the use of the wheat buyer. Shollenberger and Coleman (12) found a very definite relationship between kernel

<sup>3</sup> Reference is made by number (italic) to "Literature cited," p. 1151.

<sup>4</sup> Since this article went to press the following paper has been published: COLEMAN, D. A., DIXON, H. B., and FELLOWS, H. C. A COMPARISON OF SOME PHYSICAL AND CHEMICAL TESTS FOR DETERMINING THE QUALITY OF GLUTEN IN WHEAT AND FLOUR. *Jour. Agr. Research* 34: 241-264. 1927.

texture and protein content when climatic, cultural, and varietal influences were held constant.

As to the relation of test weight per bushel to protein content, Snyder (15) found that light weight and shrunken kernels are deficient in starch but comparatively rich in total protein; especially was this evident when the seeds were taken from the same source. Bailey and Hendel (1), Davidson and LeClerc (3), Mangels and Sanderson (9), and Mangels (8) found no significant relationship between test weight per bushel and protein content.

#### METHODS OF STUDY EMPLOYED

So far as known, all previously published results concerning the relationships of kernel texture and test weight per bushel to protein content obtained by correlation studies were derived by gross linear correlation methods; that is, the effect of only one factor at a time was considered, and no attempt was made to determine whether that effect varied in different parts of the range. In the present paper the study of these relationships has been extended, and the results given were derived from the use of net and multiple linear and multiple curvilinear methods. These methods make it possible to consider the effect of several factors working simultaneously and to allow for differences in the effect of each factor in different portions of its range. Moreover, these methods are usually more accurate and complete in their indication of relationships. In cases where the relationship is not linear or where there is a reversal of direction, curvilinear correlation methods are particularly valuable.

#### SOURCE AND DESCRIPTION OF DATA

The correlation studies here presented were made from 1,290 samples of hard red spring wheat grown during the crop years 1915 to 1923, inclusive, in 20 Northern and Western States, a few in Canada, and two in Manchuria. Slightly over 60 per cent were produced in the four largest spring wheat-producing States, North Dakota, South Dakota, Minnesota, and Montana. About half of the samples were obtained from commercial lots of grain and the remainder from experiment station plots.<sup>5</sup>

In Table 1 data are given which show by crop year the number of commercial and experimental samples obtained from each of the various geographical sections.

Most of the experimental samples were grown in variety tests by the Office of Cereal Investigations, Bureau of Plant Industry, in cooperation with the various State experiment stations. Twenty-nine varieties are represented in the samples, of which 255 samples were of Marquis and 98 of Kota. In the case of the commercial samples it seems probable, although it has been impossible to ascertain with certainty, that the greater portion of the commercial samples were of Marquis. The commercial wheats were obtained chiefly from Federal grain supervision offices located at the larger grain markets, and are believed to be fairly representative of the hard red spring wheats marketed during each of the respective seasons. Data regarding the varieties of wheat included in the samples are presented in Table 2.

<sup>5</sup> The method employed in securing representative samples is fully described in U. S. Department of Agriculture Bulletin No. 1187 (13).

TABLE 1.—Number and source of samples from hard red spring wheat grown 1915-1923

Source	1915	1916	1917	1918	1919	1920	1921	1922	1923	Total
	Number of experimental samples	Number of commercial samples	Number of experimental samples	Number of commercial samples	Number of experimental samples	Number of commercial samples	Number of experimental samples	Number of commercial samples	Number of experimental samples	Number of commercial samples
Arizona										1
California			2	2	1		2		1	0
Canada									10	0
Colorado					2	1			0	36
Idaho			2	2	1	4		5	5	35
Iowa							1	2	2	14
Kansas								10	1	1
Manchuria							3	3		0
Michigan				1		2				0
Minnesota										1
Montana			5	3	9	5	22	28	11	63
Nebraska			11	10	9	18	9	28	35	138
North Dakota		1	11	4	1	3	10	4	10	36
Ohio			27	21	13	11	25	26	29	163
Oregon			53	2				6		31
South Dakota			4	33	3	7	12	4	11	68
Utah			22	8	6		1	13	14	38
Vermont			1	2	2	1	3	1		9
Washington										0
Wisconsin			1	2	2	2	6	3	6	24
Wyoming								2		2
Unknown			14	17	5	14	5	4	8	77
Total	0	1	11	0	55	32	65	127	122	686

TABLE 2.—*Number of samples of each variety of hard red spring wheat grown 1915-1928*

Variety	1915		1916		1917		1918		1919		1920		1921		1922		1923		Total
	Number of experimental samples	Number of commercial samples	Number of experimental samples	Number of commercial samples	Number of experimental samples	Number of commercial samples	Number of experimental samples	Number of commercial samples	Number of experimental samples	Number of commercial samples	Number of experimental samples	Number of commercial samples	Number of experimental samples	Number of commercial samples	Number of experimental samples	Number of commercial samples	Number of experimental samples	Number of commercial samples	
Chul					5														5
Converse													1						1
Early Triumph																			3
Erivan									1				1				1		2
Gbirka					4		3		2		1		1						3
Glyndon					10		4		2				1						12
Haynes Bluestem	1	1			12	4	9	5	5		5		4						18
Huston						2					1						5		53
Iobred																			3
Java																	1		1
Kitchener							6		3		1						1		2
Kota							2		1		1						4		24
Ladoga													25				19		98
Laramie												1					1		3
Marquis			4		18	59	17	36	13		1		30				25	3	255
Norka							1				17		5		33				10
Pioneer					11		6		6		2		2		1		1		31
Power					14		11	1	7		6		6				1		55
Prelude					11		1		3		5		3		3		7		23
Preston			2		14	13	10	1	9		6		7		5		6		73
Progress																	1		1
Red Bob																	13		43
Red Fire						3	1		1		4		2		12		1		10
Redsask														1			6		10
Reliance														3			1		1
Ruby																	1		1
Rysting											4		15		9		9		38
Unknown			4		2		1	13	1	2			1						1
Probably Marquis						14		44		30		65	6	65	8	105	19	130	56
Total	0	1	11	0	101	95	73	100	55	32	66	65	131	68	127	110	122	133	1,290

Except in test weight per bushel all wheats included in this study met or bettered the requirements of the official standards of the United States for No. 2 grade of hard red spring wheat. The determinations for texture of kernel, test weight per bushel, and other factors used in establishing the grade of these samples were made in accordance with the rules and instructions under which licensed inspectors are required to grade grain.

TABLE 3.—*Correlation between kernel texture and protein content of hard red spring wheat*

Protein content *	Number of samples containing specified percentage of dark, hard, and vitreous kernels																				
	0.1-5 per cent	5.1-10 per cent	10.1-15 per cent	15.1-20 per cent	20.1-25 per cent	25.1-30 per cent	30.1-35 per cent	35.1-40 per cent	40.1-45 per cent	45.1-50 per cent	50.1-55 per cent	55.1-60 per cent	60.1-65 per cent	65.1-70 per cent	70.1-75 per cent	75.1-80 per cent	80.1-85 per cent	85.1-90 per cent	90.1-95 per cent	95.1-100 per cent	Total
Per cent:																					
7.....	1	2				3							1								4
8.....	1	1				2							2								5
9.....	1	1				2				2			2								27
10.....	2	4	2	2	2	5	2	2	2	3	12	9	9	5	4	5	8	4	3	3	101
11.....	3		1	1			2	1	7	9	5	11	16	16	16	35	25	23	24	10	210
12.....		1	1	1		2	4	6	3	12	9	9	3	9	13	29	34	39	29	34	221
13.....	2	1			1	1		1	1	2	2	2	3	2	5	24	22	32	50	53	209
14.....		1		1					1	2	1	1	3	1	2	6	9	11	24	52	79
15.....			1	1					2							3	4	6	7	28	64
16.....		1										1			1	5	8	16	51	85	
17.....			1	1										2		2	2	3	16	44	70
18.....																2	2	1	5	21	31
19.....																	4		1	5	6
20.....																				2	2
Total...	10	12	5	7	5	12	18	15	18	31	27	38	30	46	51	114	117	141	228	365	1,290

$$M_K = 80.6 \pm .3925 \quad \sigma_K = 20.9 \pm .2775$$

$$M_P = 13.6 \pm .0432 \quad \sigma_P = 2.3 \pm .0305$$

\* Basis 13.5 per cent moisture.

TABLE 4.—*Correlation between test weight per bushel and protein content of hard red spring wheat*

Protein content *		Number of samples of specified test weight per bushel																					Total
		43-43.9 pounds	44-44.9 pounds	45-45.9 pounds	46-46.9 pounds	47-47.9 pounds	48-48.9 pounds	49-49.9 pounds	50-50.9 pounds	51-51.9 pounds	52-52.9 pounds	53-53.9 pounds	54-54.9 pounds	55-55.9 pounds	56-56.9 pounds	57-57.9 pounds	58-58.9 pounds	59-59.9 pounds	60-60.9 pounds	61-61.9 pounds	62-62.9 pounds	63-63.9 pounds	
Per cent:																							
7.....																			2	1	1		4
8.....																			2	2			5
9.....																			3	8	1		27
10.....				1	1														21	11	1		101
11.....						1				1	1								22	11	1		210
12.....		1					1	1	1	1	3	3							43	31	7		221
13.....				2				2	2	3	3	3	6	12	18	19	23	35	22	33	22	1	209
14.....					2			1	1	1	1	3	4	4	21	17	27	27	23	27	24	2	193
15.....						1	1				1	4	2	3	3	7	10	9	19	18	10		116
16.....											2	3	3	6	10	8	12	14	9	5	4		85
17.....											3	3	6	8	3	17	13	4	9	3	1		70
18.....							1		1			4	4	3	3	3	2	6	3	2			31
19.....											1	1	3	3	3	3	2						16
20.....								1					1										2
Total.....		1	0	3	1	7	3	7	16	12	23	42	50	68	88	128	155	173	190	179	121	20	31,290

$$M_T = 58.6 \pm .060 \quad \sigma_T = 3.2 \pm .0425$$

\* Basis 13.5 per cent moisture.

The crude protein ( $N \times 5.7$ ) percentages used in connection with these samples are on the basis of a 13.5 per cent moisture content. The determination of this factor for the wheat samples from the crops since 1918 was made by the chemical research laboratory of the Grain Division, Bureau of Agricultural Economics.

Tables 3, 4, and 5, respectively, show the distribution of kernel texture with protein content, the distribution of test weight with protein content, and the distribution of kernel texture with test weight.

TABLE 5.—Correlation between kernel texture and test weight per bushel in hard red spring wheat

Number of samples containing specified percentage of dark, hard, and vitreous kernels																						
Test weight per bushel	0.1-5 per cent	5.1-10 per cent	10.1-15 per cent	15.1-20 per cent	20.1-25 per cent	25.1-30 per cent	30.1-35 per cent	35.1-40 per cent	40.1-45 per cent	45.1-50 per cent	50.1-55 per cent	55.1-60 per cent	60.1-65 per cent	65.1-70 per cent	70.1-75 per cent	75.1-80 per cent	80.1-85 per cent	85.1-90 per cent	90.1-95 per cent	95.1-100 per cent	Total	
Pounds.																						
43																				1	1	
44																					0	
45																			1	2	3	
46										1											1	
47										1											7	
48												1									3	
49													1								7	
50											1			1							16	
51																1	1				12	
52	1										1				2		1				23	
53	1	1		2												2	6	4		10	42	
54			1											2		2	6	7		19	50	
55							2	2		2		5	2	1	1	3	6	8		20	88	
56	2	1	1	2		1	1	1	1	1	3	1	1	2	2	6	9	13	14	31	88	
57		1	1			1	1	1	2	1	2	1	1	6	8	16	12	16	17	35	128	
58	2	1				1	1	1	4	4	2	2	2	7	6	13	20	14	33	42	155	
59	2	3			2	1	2			3	1	4	3	6	9	10	22	11	18	31	173	
60	2	3	1	1		3	3	6		3	9	6	8	4	7	7	18	19	13	32	45	190
61		1			2	5	3	2		2	3	8	7	8	7	6	11	13	19	38	42	179
62				2	1	1	4	2		3	5	2	2	4	3	5	7	17	19	38	121	
63											1	1				1		3	6	5	20	
64							1							1				2			3	
Total	10	12	5	7	5	12	18	15	18	31	27	38	30	46	51	114	117	141	228	365	1,290	

## CORRELATION OF KERNEL TEXTURE AND TEST WEIGHT WITH PROTEIN CONTENT

The correlation studies of the relationships of the two grading factors kernel texture and test weight to protein content were considered first by gross linear correlation methods,<sup>6</sup> followed by a study employing multiple linear correlation methods (16), and a study of these relations by multiple curvilinear methods (4).

In Table 6 are given the correlation coefficients and the standard errors of estimate for gross linear correlation, and the correlation coefficients, regression coefficients, and the coefficients of determination (14) for net linear correlation found by correlating kernel texture and test weight per bushel with the protein content.

In Table 7 are given the coefficient and index of correlation, standard errors of estimate, and the joint determination of protein content, kernel texture, and test weight as determined by multiple

<sup>6</sup> The coefficients and the standard errors of estimate for gross linear correlation were determined by the use of double frequency tables as described by Richey (10)

linear and multiple curvilinear correlation studies. Neither the multiple linear correlation coefficient nor the multiple curvilinear correlation index given in this table can properly be preceded by a plus or minus sign to denote the nature of the correlations because they measure a joint relationship in which the correlation between certain of the factors may be positive and between other factors negative.

TABLE 6.—*Gross and net linear correlation of protein content with kernel texture and test weight*

Item	Gross correlation		Net correlation		
	Coefficient	Standard error of estimate <sup>a</sup>	Coefficient	Regression coefficient	Determination coefficient
Protein content correlated with—					
Kernel texture.....	+0.5363±0.0134	1.92	+0.5310	+0.0564	<i>Per cent</i> 27.8
Test weight.....	-0.2315±0.0178	3.09	-0.2147	-0.1300	4.2
Total.....					32.0

<sup>a</sup> In percentage points of protein determination.

TABLE 7.—*Multiple linear and multiple curvilinear correlation of protein content with kernel texture and test weight*

Item	Multiple linear correlation			Multiple curvilinear correlation		
	Coefficient	Standard error of estimate	Determination coefficient	Index	Standard error of estimate	Determination coefficient
Protein content correlated with kernel texture and test weight.....	0.5661	1.87	<i>Per cent</i> 32.0	0.6627	1.7	<i>Per cent</i> 44.0

<sup>a</sup> In percentage points of protein determination.

The gross correlation coefficient found between kernel texture and protein was +0.5363, indicating a fairly high positive relationship, whereas the gross correlation coefficient between test weight and protein was -0.2315, indicating for these two latter factors a significant negative relationship, although not so close a relationship as that between kernel texture and protein content.

The study of these factors by net linear correlation methods gave +0.531 as the net coefficient of correlation between kernel texture and protein content, and -0.2147 as the net coefficient of correlation between test weight and protein content. These net coefficients indicate practically the same degree of relation as shown by the gross coefficients, and, furthermore, denote that there is no intercorrelation between test weight and kernel texture affecting the individual relations between these two factors and protein. Analysis by means of multiple linear correlation between these two factors and protein gave 0.5661 as the coefficient of multiple correlation, indicating that these two factors combined accounted for 32<sup>7</sup> per cent of the varia-

<sup>7</sup> This figure is derived by squaring the coefficient of multiple correlation (R<sup>2</sup>), and represents the proportion of the total variability for which kernel texture and test weight are responsible in this particular combination of factors.

tion in protein content based on the assumption that the relationship is linear. A test for curvilinearity by the approximation method (4) revealed, however, that the relationship of both factors to protein was decidedly curvilinear in character. Curves were determined by the curvilinear correlation method to show the net effect of kernel texture upon protein (fig. 1) and the net effect of test weight upon protein (fig. 2). Computing the multiple correlation for these curves

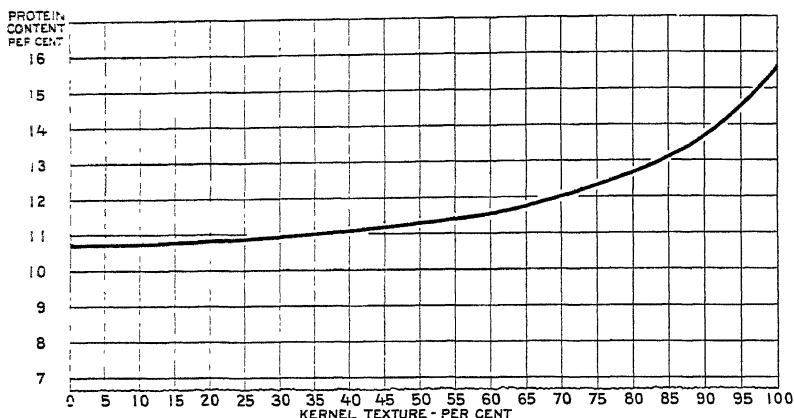


FIG. 1.—Net relation of kernel texture to protein content. This curve shows for hard red spring wheat the differences in protein content due to certain differences in kernel texture. Test weight per bushel was held constant by mathematical methods

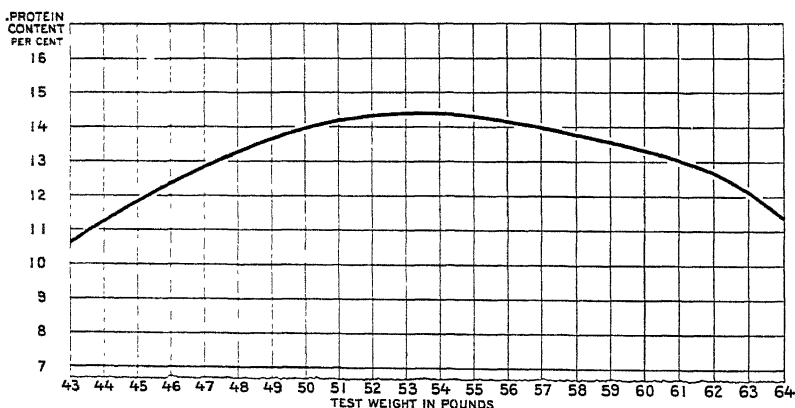


FIG. 2.—Net relation of test weight per bushel to protein content. This curve shows for hard red spring wheat the differences in protein content due to certain differences in test weight per bushel. Kernel texture was held constant by mathematical methods

gave a correlation index of 0.6627. This is a considerable increase over the coefficient of multiple linear correlation, 0.5661, showing that there is a marked curvilinearity in the relationships. The joint coefficient of determination for the curvilinear relations is 44, indicating that, when the relations are considered, curvilinear kernel texture and test weight combined account for 44 per cent of the variations in protein content. Since the joint coefficient of determination for the curvilinear relations is 12 points higher than the

joint coefficient for linear correlation, it is evident that the determination of variability of protein has been increased 12 points by curvilinear methods, and that the curves shown in Figures 1 and 2 more accurately describe the relationships of kernel texture and test weight to protein content than would the regression lines of linear correlation.

Although nearly half—44 per cent—of the variability of protein content was due to the influence of kernel texture and test weight, there still remains 56 per cent of the variations which are due to the influence of other conditions or factors. These other factors have not been considered in this article.

#### RELATION OF KERNEL TEXTURE TO PROTEIN CONTENT

In the correlation studies here reported there was found a fairly strong tendency for protein content to increase as the percentage of dark, hard, and vitreous kernels increased.<sup>8</sup> This tendency, however, was not constant, but was more pronounced among the high percentage of dark, hard, and vitreous kernel groupings than among the intermediate and low percentage groupings of samples. This relationship between kernel texture and protein content is shown in Figure 1. From this graph it may be seen that in the range from 75 to 100 per cent of dark, hard, and vitreous kernels the increase in protein content for each 1 per cent increase in dark, hard, and vitreous kernels is approximately twice as great as that from 0 to 75 per cent. And because 75 per cent marks the dividing line in the Federal wheat standards between the Dark Northern Spring and Northern Spring subclasses of hard red spring wheat, this fact is of particular significance, for it means that the kernel texture limitations of the Dark Northern Spring subclass are about twice as significant as indicators of protein content as those of the Red Spring and Northern Spring subclasses combined.

Below 40 per cent of dark, hard, and vitreous kernels there was for each 1 per cent increase in that factor an average increase of less than 0.01 per cent in the protein content; for each 1 per cent increase between 40 and 75 per cent the average gain in protein content was slightly greater than 0.03 per cent; and for each 1 per cent increase between 75 and 90 per cent an average increase of 0.09 per cent in protein was shown. The greatest rate of increase in protein was found to accompany changes in kernel texture in wheats above 90 per cent of dark, hard, and vitreous kernels. This increase averaged 0.19 per cent gain in protein with each 1 per cent increase in kernel texture.

#### RELATION OF TEST WEIGHT TO PROTEIN CONTENT

From the correlation studies reported in this article, test weight per bushel was found to have a significant relationship to protein content. The nature of this influence, however, was not the same throughout the entire range in test weight, which extended from 43 to 64 pounds. As the test weight increased in the lower portion of the range, the percentage of protein was also inclined to increase,

<sup>8</sup> As used through this article the terms "increase" and "decrease" or "rate of change" are meant to indicate the differences in the relation of factors from sample to sample. It is obvious that a given sample of wheat can not change its protein content or its proportion of hard kernels. What is meant in the statements in the text is that samples which show the higher percentages of dark, hard, and vitreous kernels also show the higher protein contents.

but as test weight increased in the remainder of the range the percentage of protein was inclined to decrease. As can be seen from Figure 2, wheats up to 54 pounds in test weight per bushel showed about the same relation between differences in protein content and differences in test weight, while above 54 pounds per bushel the protein decreased as test weight increased, this decrease becoming even more marked in wheats weighing above 61.5 pounds.

Wheats with test weight between 43 and 54 pounds increased in protein content on an average 0.34 per cent for each 1 pound increase in test weight. On the other hand, wheats with test weight between 54 and 61.5 pounds decreased on an average 0.20 per cent in protein content with each pound increase in test weight, while wheats with test weight above 61.5 pounds decreased on an average 0.60 per cent in protein content for each pound gain in test weight.

It will be noticed that the increase in protein in wheats weighing between 43 and 54 pounds was nearly twice as great as was the decrease in protein in wheats weighing between 54 and 61.5 pounds. In connection with the decrease in protein content associated with increases in test weight per bushel above 54 pounds, it is interesting to note that up to 61.5 pounds the decrease is only one third as great as that in wheats above 61.5 pounds.

It has been generally believed that there is a negative relation between test weight and protein content; in other words, that the protein content tends to increase as the test weight decreases. Certain previous linear correlation studies have not upheld this belief, but by the application of curvilinear methods it is substantiated within certain limits. (Fig. 2.) These methods show that for wheats weighing more than 54 pounds the relation was negative; for those weighing less than 54 pounds the relation was positive. This reversal of tendency in relationship probably accounts for the lack of relationship reported by previous investigators using linear correlation methods of analysis, because the negative and positive influences operating at opposite ends of the range in test weight tend to cancel each other.

If the reversal in direction of relationship shown by these studies is correct, it should follow that the conditions responsible for lowering the test weights below 54 pounds also tend to lower the protein content, and that the conditions responsible for the test weights above 54 pounds have an opposite effect on the protein content. Because of these opposite effects it is natural to expect that the conditions responsible for them are different. That such is the case is evident from the fact that test weights below 54 pounds seldom occur except in crops injured by rust. Headden (?) reports that rust causes a very marked depression in the protein content of wheat. Therefore, since the extremely low test-weight wheats occur chiefly in the crops affected by rust, and since test weight is, in part at least, a measure of the extent of the rust injury, the correlation between test weight and protein content for wheats thus affected should properly be positive. On the other hand, it has been proved that under normally healthy conditions of growth the proteins are formed or stored in the wheat kernel at an early stage in the kernel's development and the starch is placed there during the later stages. It is the abundance of starch that gives plumpness to the kernel, and plumpness is associated with high test weight; therefore, con-

ditions which arrest the development of the kernel or shorten its period of maturity will produce a kernel of lower starch content and therefore of lower test weight per bushel with a relatively higher proportion of protein content. This, then, accounts for the negative relation between test weights above 54 pounds and protein content, and tends to prove the accuracy of the relationships here shown.

#### COMBINED RELATION OF KERNEL TEXTURE AND TEST WEIGHT TO PROTEIN CONTENT

As has been shown in previous paragraphs, both kernel texture and test weight were found to have a significant influence upon protein content. Forty-four per cent of the variations in protein were due to the combined influence of these two grading factors.

The results from the linear correlations indicate that kernel texture is much more important than test weight, and the curvilinear correlations, which give a closer approximation to the true relationship, provide evidence that test weight is really considerably more important than was indicated by the linear correlation coefficients. Computation of the coefficients of net curvilinear correlation is a long and tedious operation and was not undertaken.

As previously stated, variations in kernel texture and test weight accounted for 44 per cent of the variations in protein; which means that there still remain 56 per cent of the variations unaccounted for. Undoubtedly the larger portion of these undetermined variations are due to differences in varietal tendencies, soil, cultural methods, quantity of fertilizer, and time of applying it, rainfall, and other growing conditions. These same causes of variation very likely have a direct influence also upon the kernel texture and the test weight. This being the case, these undetermined influences will, through kernel texture and test weight, affect the protein content indirectly as well as directly. What these other influences are and what their importance is, have not been considered in this study.

#### ESTIMATION OF PROTEIN CONTENT

In the hard red spring and hard red winter wheat-producing sections of this country the price received for wheat by both the country shipper and the terminal-market grain merchant is directly affected by the protein determination made at the terminal market. The farmer, except when he markets his wheat himself or through some cooperative agency, receives no direct benefit from the determination of this factor. There is, however, an indirect way in which the price benefits resulting from protein content may be reflected back to the farmer. Returns from the early shipments for any season may indicate to the country shipper the protein-content tendency in the wheat grown in his locality, and thereby influence the general level of price offered for later deliveries of wheat at that point.

Variations in such factors as soil type and condition, rainfall, elevation, atmospheric temperature, seed variety, seed-bed preparation, and land drainage are responsible for the differences occurring in protein content, and since these factors do not vary as much over small as over large areas, protein returns on a few shipments of wheat are indicative, to some extent at least, of the protein content of other wheat of the same crop produced in the same locality. For a number of years the grain trade has given recognition to this fact by

paying given premiums for wheat that comes from certain shipping points. This is known as "map" buying, and, although it is not always an accurate guide to quality, it serves this purpose in the absence of any better means of judging quality. Under this system the price reflected back to the farmer is based upon the average quality of previous receipts of wheat during that season from his locality. It will be seen that benefits based on this principle may prevent the producer of excellent-quality wheat from obtaining the full market value of his product, and may give to the producer of low-quality wheat more than he is entitled to receive.

Only when the country shipper can know, either through actual test or through some reliable method of estimation, the milling and baking value of the wheat offered to him can the individual producer of high-quality wheat expect to receive the full benefit of the quality of his product. Any means, therefore, by which the shipper can know or more accurately estimate quality will aid in bringing this about. Since protein content has been so generally accepted by the grain trade as an indicator of quality and is being so extensively used for determining the price commensurate with the quality that the wheat is assumed to have, any means whereby the protein content may be estimated by the country shipper would accomplish this purpose.

TABLE 8.—Average net relation between percentage of dark, hard, and vitreous kernels (kernel texture) and percentage of protein in samples of wheat

Percent- age of dark, hard, and vitreous kernels (kernel texture)	Percent- age of protein content	Percent- age of dark, hard, and vitreous kernels (kernel texture)	Percent- age of protein content	Percent- age of dark, hard, and vitreous kernels (kernel texture)	Percent- age of protein content
0	10.7	35	11.0	70	12.0
5	10.7	40	11.1	75	12.3
10	10.7	45	11.2	80	12.7
15	10.8	50	11.3	85	13.1
20	10.8	55	11.4	90	13.7
25	10.9	60	11.5	95	14.5
30	10.9	65	11.7	100	15.6

TABLE 9.—Correction of estimated protein content to take account of differences in test weight

Test weight per bushel	Correc- tion factor to the protein content given in Table 8	Test weight per bushel	Correc- tion factor to the protein content given in Table 8	Test weight per bushel	Correc- tion factor to the protein content given in Table 8
<i>Pounds</i>	<i>Per cent</i>	<i>Pounds</i>	<i>Per cent</i>	<i>Pounds</i>	<i>Per cent</i>
43	-2.7	51	+0.9	59	+0.3
44	-2.0	52	+1.0	60	.0
45	-1.5	53	+1.1	61	-.2
46	-.9	54	+1.1	62	-.6
47	-.4	55	+1.0	63	-1.2
48	+.0	56	+1.9	64	-1.9
49	+.4	57	+1.7		
50	+.7	58	+1.5		

From the relationship of kernel texture and test weight per bushel to protein content, which the multiple curvilinear correlation studies reported in this paper have shown, it would appear that a fairly reliable estimation of protein content can be made if kernel texture and test weight per bushel are known. Based on these studies, such a method of estimation is here presented.

In making the estimations of protein content, Tables 8 and 9 are employed. These tables were compiled from the net regression curves shown in Figures 1 and 2.

In Table 8 are presented estimates of the protein value most likely to accompany certain given kernel textures, and in Table 9 are presented correction factors for taking into account the influence of test weight upon protein content. In estimating protein content Tables 8 and 9 are used in the following manner:

Example 1: What percentage of protein in a lot of hard red spring wheat is indicated by a sample of 62 pounds test weight, containing 50 per cent of dark, hard, and vitreous kernels, when the effect of both of these grading factors upon protein content is considered?

Solution: In Table 8, column 1, find 50 per cent kernel texture, read from column 2 the accompanying protein value, which is 11.3 per cent. In Table 2, column 1, find 62 pounds test weight and then, from column 2, read the correction factor, which is  $-0.6$ . Make the correction by subtracting 0.6 from 11.3 per cent, which gives 10.7 per cent as the percentage of protein most likely to accompany wheat containing 50 per cent of dark, hard, and vitreous kernels and having a test weight of 62 pounds. In case the correction factor is preceded by a plus sign the estimated protein content is obtained by adding the correction factor to the protein value taken from Table 8.

#### RELIABILITY OF PROTEIN ESTIMATES

On the basis of the samples used in this study, if one should employ these tables to estimate the protein content of wheat when the kernel texture and test weight are known, the probable error of the estimates would be  $\pm 1.1$ ; that is, one-half the estimates should be within 1.1 per cent lower or higher than the actual protein content. The standard error of estimate would be  $\pm 1.7$ , and 68 per cent of the estimates should be within 1.7 per cent lower or higher than the actual protein content. That is, in 68 per cent of the samples, if the actual protein percentage was more than the estimate, it did not exceed it by more than 1.7, and if it was less than the estimate, it did not drop more than 1.7. These calculations are based on a uniform moisture content in the wheat.

The kernel textures of the samples used in this study represent the percentage by weight of kernels which were wholly dark, hard, and vitreous in appearance. Kernels of a vitreous character that had starchy spots as well as those that were wholly starchy in appearance were not considered dark, hard, and vitreous. This is in accordance with the interpretation placed on this term as ordinarily used in connection with the inspection and grading of wheat. In this interpretation no account is taken of the relative differences in texture between kernels partly vitreous or wholly vitreous but of a bleached appearance and those wholly starchy in character; and to that extent texture analyses of samples based on this interpretation are not a true indication of kernel texture except in cases where no mottled or bleached kernels are present. Judging from the relationship

found between kernel texture and protein content in connection with this study it is reasonable to believe that if the analysis for kernel texture in these samples had been based on a method more indicative of the true relative hardness of the kernels an even closer relationship would have been apparent, and that the difference existing between the estimated and the actual protein contents would have been materially decreased.

#### CORRELATION OF KERNEL TEXTURE AND PROTEIN CONTENT WITH TEST WEIGHT

The relationship of kernel texture and protein content to test weight was approached by gross and multiple linear correlation methods. The results of these studies are given in Table 10. Since the correlation of test weight to protein content has already been discussed it need not be considered further.

In correlating kernel texture with test weight a gross coefficient of  $-0.0953$  and a net coefficient of  $+0.0351$  were found. These insignificant coefficients show no correlation between these two grading factors.

TABLE 10.—*Gross and net correlation of kernel texture and protein content with test weight*

Item	Coefficient of gross correlation	Coefficient of net correlation	Coefficient of multiple correlation
Test weight correlated with—			
Kernel texture.....	$-0.0953 \pm 0.0186$	$+0.0351$	} 0.234
Protein content.....	$-0.2315 \pm 0.0178$	$-0.2147$	

#### SUMMARY

Data from 1,290 representative samples of hard red spring wheat were studied by gross, net, and multiple linear correlation methods and by multiple curvilinear methods to determine the relationship of kernel texture, test weight, and protein content.

A fairly strong tendency was noted for protein content to increase as the percentage of dark, hard, and vitreous kernels increased. This relation was curvilinear in character, and was pronounced in the samples having a high percentage of dark, hard, and vitreous kernels.

A significant correlation was found between test weight and protein content. The tendency was for protein to increase as the test weight increased in wheats weighing less than 54 pounds, but in wheats weighing more than 54 pounds the tendency was for protein to decrease as the test weight increased.

Forty-four per cent of the variation in protein was found to be due to the combined influence of these two grading factors. Kernel texture, however, was considerably more important than was test weight.

A method is presented for estimating the protein content when the kernel texture and test weight are known. By the use of this method the estimated protein content of 68 per cent of the samples would be within 1.7 per cent of the actual protein value.

No correlation was found between test weight per bushel and kernel texture.

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